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INFECTION OF CHICK EMBRYOS WITH NON-PIGMENTED FORMS OF *PLASMODIUM GALLINACEUM*

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Infection of chick embryos with *Plasmodium gallinaceum* has been accomplished by allowing mosquitoes to feed on the chorio-allantonic blood vessels (Haas and Ewing, 1945) and by intravenous inoculation of parasitized blood (Haas, Feldman, and Ewing, 1945). A third method, employing injection into the yolk sac of an emulsion of brain containing exo-erythrocytic forms, has been discussed briefly in a previous report (Haas, Wilcox, Davis, and Ewing, 1945). Further observations on the exo-erythrocytic type of infection produced by this method of inoculation are presented herein.

Serial passage of the infection: A chicken brain infected with exo-erythrocytic forms of *P. gallinaceum* was made into an emulsion with 10 cc. of physiological saline solution and inoculated into the yolk sacs of 9 day old embryos. From 10 to 12 days later exo-erythrocytic forms were found in the brain, liver, and spleen of these embryos; only rare parasites were seen in the erythrocytes. One of these embryo brains was used to inoculate a second lot of embryos, and in this fashion the infection has been carried through 7 serial passages. The dose of brain emulsion routinely employed is either 0.3 or 0.5 cc., depending on whether the particular brain being used has a heavy or moderate infection. Sterile technique is followed throughout; a drop of penicillin added to the emulsion does not affect the parasites, and probably aids in controlling accidental bacterial contamination.

Development of infection and predominance of exo-erythrocytic forms: A considerable number of embryos died within the first 5 days after inoculation, probably because of trauma, since the *P. gallinaceum* infection did not develop rapidly enough to account for these deaths, as is evident from the data in Table 1. Although parasites were found as early as the 7th post-inoculation day in blood films, it was not until the 9th day that any appreciable number of embryos developed infection of detectable degree.

Exo-erythrocytic forms were present in every one of the 112 infected embryos, and in 14 of the 16 chicks which were found to be infected after hatching. In all the infected embryos, exo-erythrocytic forms constituted the predominant feature of the disease: parasites in the red blood cells were either absent or too rare to count

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in 80, and in the remaining 32, densities ranged from 5 to 450 parasitized cells per 10,000 erythrocytes, the mean being 116. (In embryos infected by intravenous inoculation of parasitized blood, densities of 5000 and over are common (Haas, Feldman, and Ewing, 1945)). In the 14 chicks found to have exo-erythrocytic infections after hatching, erythrocytic parasites were not found in 7, and the remaining 7 showed densities of 2000, 200, 160, 80, 30, 10, and 5 per 10,000 red blood cells.

TABLE 1

Mortality and development of infection in chick embryos inoculated with exo-erythrocytic forms of *Plasmodium gallinaceum*

Deaths and infection in 337 embryos inoculated***	Day after inoculation						
	1 - 5 incl	6	7	8	9	10	over 10
Embryos dying	76	3	8	11	29	26	84
Embryos examined for parasites*	15	5	13	14	40	24	103
Embryos found infected**	0	0	2	1	21	15	73

*Includes 144 embryos which died, and 70 which were killed for examination.
** Includes 71 embryos which died, and 41 which were killed.
***28 embryos hatched and are not included in the tabulation; 16 of them were infected.

The presence of exo-erythrocytic forms in chick embryos infected with *P. gallinaceum* is not confined to those inoculated by the method being discussed. They have been found with considerable frequency in chicks hatched from embryos infected by the bites of mosquitoes, as may be seen in Table 2, but it is also apparent from the table that these forms attained a greater prominence in the yolk sac inoculated series than was the case in the group infected by mosquito bites.*

The tendency toward a high degree of erythrocytic parasitemia, with or without appreciable infection with exo-erythrocytic forms, characterized the latter, whereas the former group tended to develop no erythrocytic parasitemia or very little, with exo-erythrocytic forms constituting the pricipal manifestation of the infection.

* The occurrence of exo-erythrocytic forms of *P. gallinaceum* in a single chick embryo was reported by Shortt *et al* (1940). They placed the salivary glands of an infected *Aedes aegypti* on the chorio-allantoic membrane, and 11 days later found numerous exo-erythrocytic forms in the brain, liver, spleen, and bone marrow, with "moderate" infection of the erythrocytes with pigmented parasites.

TABLE 2

Occurrence of exo-erythrocytic forms and of erythrocytic parasites in chicks hatched from embryos infected with *P. gallinaceum* by two methods of inoculation.

	Chicks hatched from embryos infected by	
	Mosquito bites	Yolk sac inoculations
Number of chicks hatched	19	28
Number infected	15	16
Number examined for exo-erythrocytic forms	12	16
Number showing exo-erythrocytic forms	7	14
Erythrocytic parasite counts, range	0 - 9,000	0 - 8,700*
Erythrocytic parasite counts, mean	4,648	788*
Number having no parasites in erythrocytes	1	7

* This includes the 2 chicks which failed to show exo-erythrocytic forms, but which developed parasite densities in the erythrocytes of 1,430 and 8,700 respectively. If these 2 chicks were omitted, the range for the other 14 would be 0 - 2,000, and the mean would be 177. For those of the mosquito bitten series which showed exo-throcytic forms, the range would be 0 - 8,300, with a mean of 3,565.

The exo-erythrocytic forms in the embryos of this series have been found commonly in a variety of tissues, including brain, liver spleen, lung, bone marrow, and blood. Heavily infected brains frequently showed congestion and hemorrhage on gross inspection; redness of the liver was often marked, and engorgement of the spleen was sometimes noted. These findings frequently permitted a provisional diagnosis prior to microscopic examination of smears; the exo-erythrocytic forms seen in the latter correspond with the description of these stages originally presented by James and Tate (1937 & 1938).

Non-pigmented parasites in erythrocytes: Probably the most striking feature of the infection in these embryos has been the occurrence of non-pigmented parasites in the red blood cells: all stages of intra-erythrocytic parasites, including mature schizonts, have been seen without pigment. When parasites have been present in the erythrocytes at all these non-pigmented forms have been the rule: in only two embryos out of 50 in which parasites were found in the red blood cells, were pigmented forms seen, and even in these the non-pigmented parasites predominated. In many cases, only young

trophozoites were seen, and many of these doubtless were too immature to have produced demonstrable pigment, but when blood smears from chicks hatched from embryos of this series were compared with those from chicks hatched from mosquito-bitten embryos, the absence of pigment in the former contrasted strikingly with its prominence in the latter, even in smears where the majority of parasites were young trophozoites.

All the embryos in this series, and all but one of the 14 hatched chicks which developed the exo-erythrocytic type of infection, died before the parasites in their erythrocytes had an opportunity to undergo more than one or two reproductive cycles, and therefore it has not been possible to determine whether or not pigmentation would regularly have appeared had further development gone on. However, blood from an embryo in which non-pigmented erythrocytic forms were found was inoculated intravenously into 10 chickens aged 5 to 7 days, and the erythrocytic parasites which appeared in these older chicks were of the pigmented variety routinely seen in chicks inoculated with parasitized blood; this suggests that pigment probably would have appeared in the embryos, too, had they survived longer.*

Blood from two embryos with non-pigmented erythrocytic forms was also inoculated into two lots of embryos by the intravenous route; since the blood here used as an inoculum contained both exo- and intra-erythrocytic parasites, both should have had an opportunity to develop in the inoculated embryos, although the former being much more numerous, would be expected to predominate. Most of the embryos died of the exo-erythrocytic type of infection, but 7 chicks hatched; 5 of these were infected and in all but one both pigmented and non-pigmented parasites were found in the erythrocytes. The occurrence of pigmented forms in such a relatively high proportion of chicks hatched from the intravenously inoculated embryos further supports the idea that pigment might have been expected in the embryos of the yolk sac inoculated series had they lived long enough to permit the maturation of more intra-erythrocytic parasite cycles.

The failure of these parasites to produce pigment in erythrocytes can not have been due to any deficiency of hemoglobin in the host cells, since embryos infected by mosquito bites and by inoculation with the regular blood passage strain of *P. gallinaceum* have consistently shown pigmented infections, often of the most heavy degree. The only explanation for the phenomenon must be that these

*It is interesting to note that these chickens developed the type of exo-erythrocytic infection described in a previous report (Haas, Wilcox, Davis, and Ewing, 1945). Of the 10 inoculated, 2 died during the first three days and are therefore excluded from analysis. The remaining 8 developed parasitemias of the following maximum densities: 0, 0, 5, 40, 50, 70, 100, 210. All died with exo-erythrocytic bodies in their brains.

particular parasites, during their earliest cycles in the erythrocytes, were incapable of producing pigment. This would logically be in line with the supposition that these parasites have evolved from the exo-erythrocytic forms which constitute such a prominent feature of the infection, and that they require a certain amount of time to develop the ability to produce pigment. The actual penetration of erythrocytes by merozoites from the large exo-erythrocytic schizonts has not been observed, but during these studies there have been many blood smears showing such schizonts in the process of rupturing, with merozoites scattered freely about the vicinity. In such preparations, erythrocytes harboring very small trophozoites closely resembling the merozoites have been noted in immediate proximity to the exo-erythrocytic schizonts. These observations have given rise to the impression that the merozoites from the exo-erythrocytic schizogony have accounted for the erythrocytic infections.

Also in line with this hypothesis has been the impression that many of the larger trophozoites and presegmenting schizonts seen in erythrocytes have shown a more deeply staining cytoplasm than is the rule with the usual erythrocytic forms of *P. gallinaceum*. The rather deep blue of such parasites is exactly like that noted in many of the exo-erythrocytic forms, a feature observed, in respect to these latter, by James and Tate (1938).

Survival of P. gallinaceum without dependence on development in erythrocytes: It is clear from the ability of *P. gallinaceum* to survive the 7 serial passages through chick embryos in the manner described that the invasion of erythrocytes and the characteristic developmental cycle in these cells is not necessary for existence and propagation of the parasite in this particular host. At the time of writing, the parasite has gone into its 8th passage, and shows as much vigor and reproductive potential as in its first, and possibly even more, with no change in its host cell preferences. So long as means of transmission from one embryo to another are provided, it appears now that the parasite is capable of continuing its existence indefinitely in this fashion.

In the initial report on the exo-erythrocytic type of infection with *P. gallinaceum* in chickens and in chick embryos, it was stated that two attempts had failed to carry this type of infection beyond the 5th passage in the former, and numerous initial difficulties had indicated that appreciable success with serial passage might not be forthcoming in the latter (Haas, Wilcox, Davis, and Ewing, 1945). It now appears that, for the chick embryo, these difficulties have been surmounted, but any assumption that indefinite survival of the exo-erythrocytic type of infection can be accomplished in the more mature chicken must be withheld until more evidence is available than exists at present.

Summary

(1) Serial transmission in chick embryos of a form of *Plasmodium gallinaceum* infection characterized by predominance of exo-erythrocytic forms has been carried through 7 passages by inoculation of brain emulsion into yolk sacs.

(2) In addition to the predominance of exo-erythrocytic forms and the low density of erythrocytic parasites, this infection has been noteworthy for the appearance of non-pigmented parasites in the red blood cells.

(3) Observations of these non-pigmented erythrocytic forms indicate that they have developed from merozoites released by exo-erythrocytic schizonts, and that they are probably unable to produce pigment during their initial intra-erythrocytic developmental cycle, but that they acquire this ability later.

(4) The results of this study indicate the ability of *P. gallinaceum* to survive indefinite serial transmission in chick embryos with negligible invasion of the erythrocytes, and without manifesting that type of metabolization of hemoglobin which results in the production of malaria pigment.

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KNOWLESI MALARIA IN MONKEYS [1, 2, 3]

I. Microscopic Pathological Circulatory Physiology of Rhesus Monkeys During Acute *Plasmodium knowlesi* Malaria

(A Motion Picture)

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This paper is one of a series describing the results of some studies of normal and pathological circulatory physiology. The main purpose of the paper is to introduce a motion picture, a medical teaching film edited for loan to medical schools, medical societies, medical personnel of the armed forces, research groups, and similar agencies. The picture shows some of the microscopic pathological cirulatory physiology of *Macacus rhesus* monkeys during acute *Plasmodium knowlesi* malaria (Knisely, Stratman-Thomas, and Eliot, 1941). It is a 16-millimeter silent Kodachrome picture, about 1500 feet long, on four reels, and takes from sixty to eighty minutes to project. Except for three scenes of the fused quartz rod method of illuminating living structures for microscopic study (Knisely, 1934, 1936, 1937, 1938), the picture was taken through the microscope, a few scenes at 32, some at 48, most at 96 diameters magnification. Obviously when the film is projected, the projector lenses and projection distances used magnify the film images many times more. As far as we know, this is the first motion picture ever made of the course of the microscopic pathological circulatory physiology of a mammalian disease.

The film is, however, more than a simple record of some factors of the pathology of monkeys with this malaria. It records several factors of normal circulatory physiology which we believe are a necessary part of the controls for any study of normal, or of experimental pathological, circulatory physiology. The scenes from "normal," uninoculated, nembutal-anesthetized animals show that under the most nearly normal conditions which we have thus far attained experimentally:

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(1) The circulating red cells are not agglutinated.

(2) No white cells stick to the walls of small vessels (this agrees with and confirms the Clarks, 1935). The inner surfaces of the linings of small vessels are smooth and clean.

(3) The flow of the unagglutinated blood is laminar or streamlined.

(4) The small vessels do not leak appreciable amounts of fluid, for (a) there is no visible hemoconcentration occurring, and (b) the fat cells outside vessel walls are tight together and have flat sides where they press against each other (the cells are not being pushed and held apart by fluid leaking from small vessels, whereas when excess interstitial fluid (edema) is present it separates fat cells and permits them to round up).

(5) The blood flows so rapidly in most arterioles and venules which are from 60 to 120 micra in diameter that individual red cells cannot be seen at the magnifications used.

These five concepts of normal circulation agree with concepts of the normal derived from study of many internal organs of laboratory animals (the Clarks, 1935, Knisely, 1940) and E. H. Bloch's observations in normal unanesthetized men (*vide infra,* page 295).

The film also records several separable pathological processes which occur in rather definite sequences, combinations, and degrees of severity in monkeys with this malaria. Chief among them are (1) the massive precipitation and agglutination in the blood at the beginning of Stage III (*vide infra*), which changes all of the circulating blood to a thick, muck-like sludge; and (2) one sequence of events which this massive lethal sludging of the blood initiates.

The film shows the conditions in the small vessels of very small areas of omentum. The conditions recorded in the film have been studied at 32, 48, and 96 X with stereoscopic microscopes in all areas of the omentum, in the mesenteries, the anterior surface of the stomach, the outer surface of the small and large intestine, intestinal villi, and the peritoneal surfaces of striated body wall muscles. Most of these areas were studied in at least 50 animals.

In uninfected, nembutalized, control monkeys and in monkeys with knowlesi malaria the open capillaries of the above organs usually varied from 8 to 10 micra to about 15 micra in diameter; infrequently they dilated to 20 micra or even 30 micra. In most organs of living small laboratory mammals the open capillaries have approxi-

mately these same diameters (Knisely, 1940). This is a key point for understanding some of the pathological processes initiated by intravascular agglutination of the blood. All of the blood which does not go through sinusoids or arterio-venous anastomoses (which have thus far been found in but few tissues and organs of any one species, Clark, 1938) must pass through capillaries on each passage around the circulatory system. Thus the capillaries are a perpetual "bottle neck" in the circulatory system.

The circulation through the sinusoids of the liver lobules at the edge of the liver and the circulation in small pia-arachnoidal vessels were studied in a few animals. In the liver special phagocytic reactions were studied.

The readily observed evidences of the consistency of the flowing blood are the viscosity of the plasma and, if intravascular agglutination be present, the ranges of distribution of the sizes, the shapes, and the plasticity or rigidity, the elasticity, the toughness or fragility, and the stickiness of the clumps of coated red cells. These properties change during the course of the disease, but at any one time in flowing blood each of these properties was found to be the same in all organs observed; that is, in each organ the flowing blood is a valid sample of all the *flowing* blood in the body.

As controls for possible effects of the operations and manipulations, studies of the vessels and blood in the inner surface of the reflected eyelids of unoperated and operated monkeys were made. These control observations showed that the laparotomies were done with sufficient care to prevent general intravascular agglutination of the blood (Knisely, Eliot, and Bloch, 1945). The scenes of normal blood and vessel walls in Reel I are recorded evidence that in the hands of experienced investigators, no single factor or combination of factors of the anesthetic (nembutal), the laparotomies, or the brilliant trans-illumination of living tissues whose temperature is maintained normal, are sufficiently disturbing to the whole animal or to the tissues studied to cause the circulating blood, the white blood cells, or the vessel walls to exhibit any of the known micropscopically visible responses of these elements to injury (cf. Clark and Clark, 1935; Knisely, Eliot, and Bloch, 1945). With routine care we have been able to keep exposed tissues of nembutal anesthetized non-malarious control monkeys under continuous observation for 14 to 18 hours without causing any of the microscopially visible evidences of injury to blood or vessel walls.

The pathological circulatory physiology of *Plasmodium knowlesi* malaria in monkeys may be divided into several stages. We are calling the "prepatent period" (from the time the animal is inoculated until parasites can be detected in smears of drawn blood) Stage I. Stage II begins with the appearance of parasites and lasts until

the massive precipitation and agglutination in the blood occur. During Stage II each parasitized cell is coated with a thin layer of a precipitate which has some very special cohesive and adhesive properties. This Stage II precipitate forms on a parasitized red cell within a few moments, if not at once, when the merozoite enters it. During Stage II the monkey's unparasitized red cells are not coated with precipitate; they are smooth, clean, and unaggultinated, like any normal red cells. During Stage II the coatings on the parasitized red cells are not sticky to normal red cells. In the tissues and organs we have examined the Stage II coatings have not been sticky to normal undamaged ordinary vascular endothelium. The coating material is very sticky to itself; consequently during Stage II when circulating parasitized red cells touch each other their coatings hold them together in little clumps.

In Reel II of the motion picture the scene following title 40 and that following 41 show small clumps of parasitized red cells carried along in rapidly flowing unagglutinated blood. During Stage II the unparasitized, uncoated red cells slide and bump along the inner surfaces of the liver sinusoids and are not ingested by the phagocytic, sinusoid-lining von Kupffer cells. But when the flowing blood carries a parasitized, coated red cell or a clump of them against the stationary hepatic sinusoid lining, that coated particle and all it contains is instantly ingested by the phagocytic lining. Thus these phagocytes selectively ingest this particular coating material and inescapably take in whatever the coating contains. In the liver, during Stage II, this selective ingestion of the selectively coated parasitized red cells is very rapid. In the hepatic sinusoids which we have watched, the coated parasitized cells were removed from the columns of flowing blood cells so rapidly that the flow through the sinusoids was not impeded at all (cf. Knisely, Bloch and Warner, in preparation).

At present it seems probable that these factors of Stage II are present and operative in Stage I, for during the prepatent period great numbers of parasitized red cells are phagocytized in liver, bone marrow, and spleen (Taliaferro and Cannon, 1936, and Taliaferro and Mulligan, 1937). Further, the criterion for saying that the animal has progressed from Stage I to Stage II is not any sharply marked change in its physiology; it is arbitrary, based only on our ability to find parasites in smears of drawn blood.

Stage II may last several days. But when, during some segmentation period, the parasite count rises suddenly (usually to more than 10 percent but less than 30 percent of the animal's red cells) there is a sudden change in the consistency of the circulating blood. During a short, usually ten to twenty minute period, a new precipitate is formed throughout all the circulating blood. It is formed

between and around all the red blood cells, normal as well as parasitized. This second precipitate is a real, visible precipitate. In the film, the scene following title 48a shows a highly magnified view of some of this material under dark field illumination. The scene shows red cells imbedded in shiny, fluffy, cottony precipitate. This second precipitate coats all of the blood cells and binds them into great tough wads and masses (not rouleaux). The formation of this precipitate changes all the animal's blood to a thick, muck-like sludge and initiates Stage III of the pathological circulatory physiology.

The formation of the second, Stage III, precipitate has three important results:

(1) The large pasty masses of coated agglutinated blood cells resist passage through small vessels and thus retard the flow of blood through each tissue, thus causing progressive stagnant anoxia all over the body.

(2) The presence of this precipitate reduces the rates of phagocytosis of parasitized red cells and thus decreases the rates of destruction of parasites.

As soon as the precipitate is formed the large masses, which consist of mixtures of parasitized and unparasitized red cells, resist passage through hepatic sinusoids. This decreases the rates at which parasitized red cells can be carried to the stationary hepatic phagocytes. Further, most of the masses are too large to enter a phagocyte readily. Thus as soon as the Stage III precipitate is formed these two factors operate to reduce the rate of phagocytic destruction of parasites.

(3) The preciptate encloses all the animal's unparasitized red cells, which permits them to be phagocytized in spleen, liver, and bone marrow. This causes rapid severe blood destruction.

As Stage III progresses, many of the large clumps are forced against the sharp inner edges of the forks of arterioles and cut into fragments. These fragments contain mixtures of parasitized and normal red cells, are coated with the precipitate, and are small enough to enter hepatic phagocytes. Thus as the large clumps are broken up the rate of ingestion of coated clumps accelerates. Now, however, normal as well as parasitized red cells are included in the precipitate, and are ingested. But since the total capacity of the phagocytes is limited, and since this capacity becomes partially occupied by normal erythrocytes, the final result is a decreased rate of destruction of para-

sites and a substantial destruction of normal red cells. Thus an accelerated and severe anemia occurs.

These three factors alter the whole course of the disease.

The film shows that when all the blood is changed into a thick sludge, and each mass of precipitate-coated, clumped red cells is large enough and rigid or tough enough to resist strongly its own passage through small vessels, the flow through small vessels becomes progressively slower. In all cases, the flow becomes slower than the normal rate for each degree of dilatation of each vessel. As the rates of flow decrease, the rates of supply of oxygen to tissues become less than the normal rates of use. This causes a prolonged, maintained, and progressive stagnant anoxia of most tissues and organs of the animal's body.

The difference in pressure between the arterioles and venules of an organ, together with the number of arterioles which are dilated per unit volume of tissue, the degree to which each is dilated, the number of capillaries which are open per unit volume of tissue, the viscosity of the plasma, the number of red cells per cubic millimeter of blood, and, if present, the resistance to flow offered by agglutinated blood cells, determine the rates of blood flow through the tissues of each organ.

A cell cannot use a molecule until that molecule reaches that cell. Consequently, a stationary cell cannot use a row of molecules any faster than the row comes to the cell. Hence the factors listed in the above paragraph set the maximum possible rates at which the cells of each organ can be supplied with molecular oxygen, glucose, and other anabolites. For each substance in the arterial blood, these anatomical, physiological, and physical factors set the maximum molecular or ionic concentration which can be maintained at each point throughout the length of each capillary of each tissue. The concentration of each substance at each point along a capillary determines the concentrations at the upper ends of the radial diffusion gradients for that substance into the neighboring tissue. (For more extensive statements of these concepts see Krogh, 1929, Lecture XII; Barcroft, 1925; and I. Bloch, 1941, 1943). Thus these circulatory factors from moment to moment continually, under all normal and pathological conditions, set the maximum possible rates at which the cells of each organ can receive oxygen, glucose, and other anabolites. Obviously they also set the maximum rates at which the metabolites of each tissue can be removed. (I. Bloch, 1943).

As noted, the Stage III precipitate coats the unparasitized red cells, as well as the previously coated parasitized ones, binding them all together in wads and masses. In many animals most of the masses thus formed are too large to enter the phagocytes of the liver.

By closely spaced total and differential parasite counts we learned that at such a time the rate of phagocytosis of parasitzed red cells is greatly decreased. Later as the large masses begin to be broken up, their fragment are still coated, still contain *unparasitized* as well as parasitized red cells but are now small enough to enter liver phagocytes. In the film the scenes between titles 47 and 59 show that the circulating clumps are partly broken up. The phagocytes of liver, spleen, and bone marrow ingest these coated smaller clumps, which contain unparasitized red cells, rapidly. (cf. Taliaferro and Cannon, 1936, and Taliaferro and Mulligan, 1937).

Throughout the course of the disease (Stages I, II, III, etc.) the destruction of parasitized red cells by (a) segmentation of mature parasites and (b) the phagocytosis of coated parasitized red cells in the liver, spleen, and bone marrow, acts toward causing reduced red cell counts (anemia), and low circulating blood volume. (The number of red cells in the body at any moment is equal to the summation of the rates of production up to that moment, minus the summations of the rates of destruction. The ingestion of one coated red cell decreases (a) the number of red cells in the body and (b) the blood volume). In Stage III, after the massive clumps have been resolved into smaller fragments, the rapid ingestion of coated unparasitized red cells becomes a major factor causing a rapid progressive decrease in the number of red cells and in the blood volume. These three processes which cause and maintain anemia and low blood volume proceed at different rates as the disease progresses, and at different rates in different animals. We have found a number of factors which affect these rates; they are too complex to include in this short introductory paper.

As the animal's blood volume decreases, the arterioles of skin, subcutaneous fascia, and striated muscles shut off tightly for longer and longer periods of time. Capillaries and small veins also constrict shut. Large veins partially constrict, which often makes it difficult to obtain blood samples. When the blood volume is sufficiently low the arterioles of striated muscles remain shut off even during and following contraction of the muscles. (In animals with normal blood volume the arterioles and capillaries of striated muscles dilate widely in response to metabolites released from the contracting muscle fibers (Krogh, 1929, pp. 61-65; Anrep, 1935, 1936; and Bülbring and Burn, 1939)). When the parasitized monkey's blood volume is sufficiently low if one cuts into a striated muscle (such as rectus abdominus) it scarcely bleeds. Thus, because of prolonged spasms of arterioles and other small vessels some tissues receive much less blood than normal or no blood for much longer periods than during any phase of normal physiology (cf. Knisely, 1940).

The phagocytic reactions and the prolonged spasms of vessels are not shown in the film.

As parasites enter red cells and develop in them they destroy the hemoglobin (Morrison and Anderson, 1942) and thereby reduce the oxygen carrying capacity of the paratisized red cells. Thus the oxygen capacity of the arterial blood is decreased by two factors, (a) anemia, and (b) the decreased amount of hemoglobin in the parasitized cells.

As a result of these processes, (a) the arterioles of some tissues are shut off for much longer than normal periods, (b) the oxygen capacity of the arterial blood is decreased, and (c), because the masses of the precipitated-agglutinated blood are larger than the diameters of the small vessels, in each tissue the rates of flow through those small vessels which at any moment are open, is forcibly reduced to much less than the normal rate for each degree of dilatation of each vessel. All of these factors act toward reducing the oxygen supply to tissues. And, as previously stated, at all times the rates of flow through the open vessels of each tissue set the maximum rate at which the cells of that tissue can possibly receive molecular oxygen, glucose, and other anabolites.

At the beginning of Stage III, as the rates of flow of the heavily sludged blood progressively decrease, the venous, downstream ends of capillary beds become anoxic before the arterial ends. As the post-capillary venules and small venules become sufficiently anoxic they begin to lose their ability to retain blood colloids. This agrees with Starr 1926, Starling, 1926, Landis, 1928, Krogh, 1929, pp. 321, 326, 335, Maurer, 1940, 1941a, 1941b, Warren and Drinker, 1942, Drinker, 1942, and I. Bloch, 1941, 1943.

As might be expected, post-capillary venules and small venules become anoxic before other parts of capillary beds, they become more anoxic than other parts of local vessel systems, they become colloid permeable first, and they become most permeable. During anoxia they are the most permeable parts of local vessel systems. When sufficiently anoxic these small vessels leak fluid rapidly. The hemoconcentration occurring as sludged blood slowly passes through leaking venules is shown in the film.

At those times during the course of the disease when the summations of the rates of red cell destruction have been greater than the summations of the net rates of fluid loss from the vascular system, the venous blood has a low red cell count (anemia); at those times when the summations of the rates of fluid loss have been the greater, the venous blood shows high red cell counts (hemoconcentration).

As a result of the reduced rates of flow and consequent anoxia of the downstream ends of capillaries, post-capillary venules, and small venules, local fluid losses and local hemoconcentrations occur

all over the body and finally, as a result of rapid local fluid losses, enormous numbers of small vessels are left plugged with stranded masses of tightly agglutinated blood cells (cf. Flexner, 1902). These stranded masses act as small thrombi. Literally hundreds of thousands of minute thrombi can be and usually are formed in this way, sometimes in individual tissues and organs, sometimes almost all over the body. They can form in a short time or over a long period. When a small venule is plugged with a stranded mass, other masses continue to come down the capillaries which drain into the venule, and are forced into the upstream ends of the venule, and forced against the upstream end of the thrombus. Fluid passes out through the anoxic venule wall and the leaking vessel is thus stuffed full of sticky impacted masses which then constitute a long tough thrombus. Parts of some of these tough thrombi then break loose, circulate with the blood and embolize other small vessels. Literally thousands of small tough emboli are formed in this way. These processes are shown in Reel III of the film.

As a result of these processes, the sum of the rates of local fluid losses all over the body probably becomes greater than the sum of rates of return of fluid to the blood by lymphatics. Hence at the same time that enormous numbers of small vessels are being embolized and/or thrombosed by stranded masses of coated agglutinated red cells, there is a progressively decreased circulating plasma volume. The stranding of clumped red cells *in* small vessels and plasma loss *from* small vessels are major factors causing a progressively decreasing circulating blood volume and venous return.

Some animals were autopsied as soon as they died. As a part of the autopsy, and to have the blood for other experiments, as much blood was recovered from these animals as could be obtained by carefully aspirating the heart chambers, the aorta, and the great veins with syringes. From 10 to 12 pound animals which had died as a result of the described processes not more than 40 to 50 cc. of blood could be obtained at the time of death.

Thus the causes of death in this malaria are, (a) reduced numbers of red cells, (b) reduced oxygen carrying capacity of parasitized red cells, (c) progressive undernourishment and anoxia of vital tissues, (d) multiple thrombosis or embolization of vital tissues (perhaps of central nervous system centers), and (e) progressively decreasing circulating blood volume. Any one of these alone could cause death. Any combination can happen. In each monkey they usually act progressively, each succeeding one being added to those which have begun before. Death in any one case probably is caused by that single cause or combination of causes which first (a) destroys some vital center or organ, or (b) decreases the rate of a necessary function (such as venous return) below that necessary

for life. Thus the film shows in some detail several parts of a set of pathological circulatory processes which when sufficiently severe, prolonged, and untreated, always kill an animal. While these processes kill, they may leave but a few or almost no discernable traces of the mechanisms causing death either at gross autopsy or preserved in histological sections. No combination of still pictures and words of ours can describe these processes as clearly as the film shows them. Further, motion pictures are the only objective method of recording visible microscopic processes while they are occurring.

The film is a part of a broad study of normal and pathological circulation. By focusing stereoscopic dissection microscopes on the vessels in the bulbar conjunctiva of unanesthetized human beings we have seen circulating agglutinated blood in patients diagnosed as having each of the three human malarias, and in a wide variety of other pathological conditions and diseases. Intravascular agglutination of the blood has been seen or demonstrated in living animals and human patients, and/or its results observed in vitro or in fixed preparations by many investigators. Among these are: Donders, 1864, Hueter, 1876, 1879a, 1879b, Flexner, 1902, Luedde, 1913, Arev, 1918, Ploman, 1920, Elschnig, 1921, Fahraeus, 1921, 1928, 1929, Freedlander and Lenhart, 1922, Ruedemann, 1933, Salsbury and Melvin, 1936, Swindle, 1937, Müller, 1937, Youngner and Nungester, 1944, Oliver-Gonzales, 1944, Mills and Dochez, 1944.

These and other investigators have studied various aspects of the phenomenon. Intravascular agglutination is sometimes called "granular flow," or authors say that the column of blood has "separated into plasma spaces and short columns of red cells." German authors frequently refer to it as "körnige stromung." Intravascular agglutination has frequently been correlated with reduced flow rates through small vessels (cf. Freedlander and Lenhart, 1922, and Salsbury and Melvin, 1936). But, that intravascular agglutination of the *circulating* blood acts as a *direct* cause of reduced flow rates and thus of anoxia and undernourishment of tissues has not been recognized. There are a number of reasons for this, two of which are worth pointing out here:

(1) The flow and diffusion mechanisms whereby capillary flow rates limit and determine the environments of the cells of tissues have not been widely known (cf. Krogh, 1929, Barcroft, 1925, and I. Bloch, 1941, 1943.) Attention has been focused on arterial pressure which is necessary but not of itself sufficient to maintain normal flow rates, i.e., to maintain adequate rates of supply of anabolites to tissues; not enough attention has been accorded the

capillary flow rates themselves.

(2) In general those who have studied normal circulating blood and normal living capillaries of animals with microscopes have not studied the circulating blood and vessel walls of living human patients, and vice versa. Clear cut concepts of the normal are of course necessary as a foundation for recognizing transitions into the pathological. The clumps of agglutinated blood cells have most frequently been seen in small veins (cf. Müller, 1937) whose internal diameter was greater than the diameter of the clumps. In these vessels such clumps are usually freely suspended, hence seeing masses in veins does not suggest that they may be too large to pass through the smallest vessels easily. The diameters of normal mammalian capillaries during life have not been generally known, and the reflected light and lens combinations usually employed to study blood and small vessels of human patients have seldom permitted observers to see (a) the vessel walls, or (b) the clumps squeezed out into sausage shapes as they are driven down into the long tapering cone-shaped arterioles, or (c) the walls of the capillaries bulge as semi-rigid masses of agglutinated cells are forced into and through them.

At Memphis during the summer of 1941 E. H. Bloch examined the blood passing through the small vessels of the bulbar conjunctiva of 50 normal medical students and student nurses, using a stereoscopic microscope with 48 and 96 X magnifications. (The surveys of the circulating blood in the organs of monkeys had shown that the flowing blood passing through any tissue is a valid sample of all the flowing blood in the body (*vide supra,* page 287). In none of these normal unanesthetized medical students and student nurses was there any agglutination of the blood cells. In about 400 human patients diagnosed as having a variety of conditions and diseases, we have seen sludged blood consisting of clumps of agglutinated red cells having every conceivable size and degree of rigidity up to those which would just barely pass through the smallest vessels (Knisely and E. H. Bloch, 1942,1945.) In general (1) in any one patient the clumps have a definite range of size and range of degree of internal rigidity, (2) the degree of retardation of blood flow is proportional to the sizes and rigidity of the clumps, and (3) whenever certain degrees of retardation are present, corresponding degrees of leaking of the post-capillary venules of the bulbar conjunctiva occur. Patients may have very small clumps and normal flow rates through small vessels and still be very ill. We have seen no patients with markedly reduced flow rates who were in good clinical con-

dition. Thus the film not only shows normal blood and vessel walls and several parts of the course of events in monkeys with knowlesi malaria, but also serves as an introduction to some of the kinds of factors of pathological physiology initiated by comparable or lesser degrees of severity of intravascular agglutination of the circulating blood. In addition to papers describing parts of these investigations we wish to present an adequate sample of the evidence itself, hence the preparation of this film for general distribution to physicians and medical scientists.

The film shows pathological processes while they are still in reversible stages, that is, in controllable stages. Autopsies and autopsy sections show the final cumulative results of all the simultaneous and consecutive pathological and reparative processes which had been going on. That is, they show the preservable, visible part of the accumulated results after some one or more sets of pathological processes have become irreversible. Obviously one large part of clinical medicine consists in the detection and evaluation, and then in the controlled minimization, retardation, stopping, and reversing of pathological processes. This film shows one set of pathological processes (a) as they develop, (b) as they proceed at sub-lethal degrees of intensity, (c) as they accumulate toward a lethal combination of factors but are still reversible, that is, while the animal's life can still be saved, and (d) as they cumulate into non-reversible stages.

The film shows that in rhesus monkeys with knowlesi malaria atabrine given by stomach tube causes or permits the large tough masses of coated agglutinated red cells to disintegrate either into individual red cells or into clumps so small and soft or plastic that they do not resist passage through small vessels and thus retard the flow of blood. When given by stomach tube, this action of the drug takes place in from three to twelve hours and restores the fluidity of the blood before the parasites have become too few to count in smears. The drug must of course be given before the occurrence of the multiple thrombosis of great numbers of small vessels of the gastrointestinal mucosa, for thrombosis of these vessels effectively retards, perhaps even stops, the absorption of the drug from the gut.

The film shows that when the blood is again normally fluid, the flow is no longer retarded. As the clumps disintegrate the blood accelerates until it flows at nearly normal rates again; as the flow accelerates the small vessels regain their ability to retain blood colloids, and the loss of fluid from small vessels with the accompanying visible hemoconcentration stops. Lymphatics continue to return lost fluid to the bloodstream; at this time the rate of fluid return becomes greater than the rates of fluid loss. The circulation becomes near enough to normal for the animal to survive and convalesce.

Quinine also has this effect on the precipitated-agglutinated blood during what we are now calling Stage III of the pathological physiology of monkeys with this malaria. These drugs probably cause decreases in the internal cohesiveness or in the tensile strength or in the solubility of the viscid precipitates which at State III of this malaria coat all the red cells and bind masses of them together in large semi-rigid clumps. As noted above, precipitated-agglutinated blood has been seen in human patients diagnosed as having benign tertian, quartan, and malignant tertian malaria (Knisely and E. H. Bloch, 1942, 1945).

We wish to point out specifically that it is too early to be absolutely certain that atabrine or quinine causes direct disintegration of the agglutinated masses (unsludging) of the blood in human malaria patients. The human parasites are not the same species as knowlesi parasites, and hence may not have the same combinations of enzyme systems, and the proteins of human blood are not specifically identical with those of monkey blood and may not react to antimalarial drugs in exactly the same manner or to the same degree as do those of experimental animals. We have not yet had an opportunity to carry out a detailed study of the changes in the blood and vessel walls of human beings during the course of human malarias before and during treatment.

There are three significant conclusions to be drawn from the film's demonstration of the unsludging of the monkey's Stage III precipitated-agglutinated blood.

1. The cohesiveness, tensile strength, or solubility of the coatings on aggultinated blood cells can be affected with therapeutic agents.
2. The disintegration of the clumped masses which make up agglutinated blood is desirable and, if the masses are large enough and tough enough, the disintegration of these masses is necessary to save life .
3. Methods and competent personnel are available to test standard and experimental therapeutic agents in living animals and men to see whether and to what degree they cause the disintegration of agglutinated masses in precipitated-agglutinated blood.

Summary

A motion picture is described which consists of four reels. They are divided into three sequences:

The first sequence, Reel I, shows (1) a little of the method of study, (2) the most nearly normal circulation we have yet been able to photograph, and then sharply contrasts this with (3) advanced pathological conditions.

The second sequence, Reels II and III, traces the most common course of the changes in blood and vessel walls from normal through

progressively increasing pathological phases to several types of fatal outcome.

The third sequence, Reel IV, shows the effect of atabrine, given by stomach tube, on the precipitated-agglutinated blood of rhesus monkeys with knowlesi malaria.

Copies of the film will be loaned free, except for transportation charges, to medical schools, medical societies, medical officers of the military services, and research groups. Requests should be sent either to Dr. M. H. Knisely, Department of Anatomy, University of Chicago, or to Dr. T. S. Eliot, Department of Anatomy, University of Tennessee, Memphis.

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ON THE HETEROLOGOUS VALUE OF ACQUIRED IMMUNITY TO *PLASMODIUM FALCIPARUM*[1]

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We have entertained the opinion[2] that acquired immunity to *P. falciparum* does not have much value against heterologous strains of this parasite. Recently we have had opportunity to perform numerous reinoculations with several strains of the parasite, and our results are presented herewith.

Our data pertain to 10 white and 21 colored patients, whose *falciparum* infections were induced by inoculations with infected blood. These original inoculations, as well as the first heterologous reinoculation, are summarized in Table 1. The different strains used were not simultaneously in our possession, so it was not possible to practice systematic reinoculation of convalescents. Of the strains employed, the Bynum, Costa, Harden, and Long strains are indigenous, and the Cuban, Mexican, Panamanian, and Trinidad are exotic. Seven of the 15 primary inoculations with indigenous strains were in white patients, and three of the 16 inoculations with exotic strains were also in whites. In two instances, following successful reinoculation, with a heterologous strain, there was not concurrent clinical activity, the results thus resembling reinoculation with a homologous strain. One of these instances followed the reinoculation of a Panamanian strain convalescent with the Mexican strain, the other was noted when the Long strain was reinoculated into a Mexican strain convalescent. However, other patients in whom the same succession was followed exhibited clinical activity.

It will be noted, either when all patients are considered together, or when the different strains are considered individually, that the original infections have been materially more severe in white than in colored patients. In white patients the infections have frequently consisted of two or even three waves of trophozoites, each with its following wave of gametocytes. In colored patients the infection has been commonly limited to one wave of each. Although it might appear that the infections produced by indigenous strains have been slightly more severe than those produced by the exotic strains, it is likely that any difference apparent is attributable to the greater proportion of white patients receiving inoculation with indigenous strains.

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2. Boyd, Mark F., Stratman-Thomas, W. K., and Kitchen, S. F. On Acquired Immunity to *Plasmodium falciparum*. Am. J. Trop. Med., 16:139 - 145, 1936.

Table 1.—Original and first heterological reinoculations of white and colored patients

Strain of Original Inoculation	Strain of Reinoculation													
	White patients						Colored patients							
	Bynum	Costa	Harden	Long	Mexican	Total	Bynum	Costa	Long	Mexican	Panamanian	Perkins	Trinidad	Total
Bynum						0		1						1
Costa			2		1	3	1						1	2
Cuban				1		1		1		2	1			3
Harden	1	1			1	3								0
Long					1	1	1			1	2	1		5
Mexican					0			2		2				4
Panamanian				2		2		1	1	3				5
Trinidad						0		1						1
TOTAL	1	1	2	3	3	10	2	3	3	6	5	1	1	21

Some characteristics of the original infections are indicated by the mean values in Table 2.

Table 2.—Salient features of the original infections

Strain	Race of Patients	Number of Patients	Mean maximum density				Clinical activity		Therapeutic interference
			All parasites		Gametocytes only		Mean days		
			Days from first par. to maximum	Per cmm.	Days from first par. to maximum	Per cmm.	First to last	Actual fever	
Bynum	Col.	1	11	19,000	22	1020	7	5	0
Costa	Wh.	3	8.7	84,820	16.7	3330	35	17	2
	Col.	2	9.0	57,050	14	620	15.5	9.5	1
Cuban	Wh.	1	11	318,800	33	5260	33	16	1
	Col.	3	7.3	110,170	14.3	6440	32.7	9.7	2
Harden	Wh.	3	8	133,470	17	2710	49	24	3
Long	Wh.	1	11	369,200	34	3240	64	40	1
	Col.	5	8.6	80,410	17.4	2100	9.8	7.2	2
Mexican	Col.	4	4.2	11,140	13.2	1220	5	3.7	0
Panama-nian	Wh.	2	8.5	119,150	32	4110	33	18.5	2
	Col.	5	6.2	44,860	13.2	340	20.4	10.2	2
Trinidad	Col.	1	9	17,500	25	10	11	6	0
All Indg. str.		15	8.9	103,950	18.1	2270	26.9	14.9	9
All Exotic "		16	6.7	83,870	17.7	2460	20.6	9.6	7
All White		10	8.9±	174,115±	23.2±	3480±	41.5±	21.6±	9
		1	.64	37,128	2.84	836	4.80	2.94	
All Colored		21	7.2±	54,857±	15.4±	1842±	15.1±	7.8±	7
		11	.61	13,337	1.13	670	3.38	1.06	

Similar characteristics of the infections produced by the same strains when heterologously reinoculated into the same patients are shown in Table 3.

Table 3.—Salient features of infections resulting from first heterologous reinoculations

Strain	Race of patient	Number of Patients	Mean maximum density				Clinical activity		Therapeutic interference
			All parasites		Gametocytes only		Mean days		
			Days from first par. to maximum	Per cmm.	Days from first par. to maximum	Per cmm.	First to last	Actual fever	
Bynum	Wh.	1	7	37,800	13	650	9	9	0
	Col.	2	7	6,070	24.5	310	3.5	3.5	0
Costa	Wh.	1	8	123,000	15	1510	7	6	0
	Col.	3	9.3	52,150	19.7	3010	7.3	4	1
Harden	Wh.	3	7.5	27,200	17.5	390	5.5	3.5	1
Long	Wh.	3	7	132,180	15.3	2720	23.3	9.0	2
	Col.	3	7.3	70,230	9.7	1030	5.7	3.7	1
Mexican	Wh.	3	9.3	15,930	21.7	420	10.3	8.3	0
	Col.	6	6.7	28,070	16.3	6800	10.3	5.2	0
Panama-nian	Col.	5	9.8	19,260	14.2	1040	6.6	4.6	0
Perkins	Col.	1	7	17,600	19	170	5	2	0
Trinidad	Col.	1	7	133,800	10	10	6	6	1
All Indig.	str.	16	7.6	63,040	16.6	1500	9.2	5	5
All Exotic	"	15	8.3	29,760	16.3	3150	8.8	5.7	1
All White		10	7.9± 0.48	65,950± 21,170	17.4± 1.26	1230± 423	12.8± 2.47	7.4± 0.97	3
Colored		21	8.0± .46	37,870± 12,025	16.0± 1.42	2810± 1339	7.2± 1.38	4.4± 0.58	3

It will be noted from this table that on heterologous reinoculation the clinical attacks in white patients have been closely similar to those observed in colored patients on first inoculation, while in the latter group their mean duration has been still further curtailed. The proportion of either racial group requiring therapeutic interference has been materially less on heterologous reinoculation. The maximum density of the parasitemia occurred after about the same interval following patency of infection in both groups, but the maximum density attained by the parasitemia although generally lower, was consistently higher in the white patients. On the other hand, colored patients on heterologous reinoculation usually produced higher densities of gametocytes than did the whites, frequently even exceeding those attained in the primary infection. Nevertheless, certain of the results exhibit paradoxical aspects. Thus, in colored patients the Mexican strain produced infections of greater

severity when used in heterologous reinoculation than when it was used in primary inoculation, although the infections resulting from the employment of this strain in heterologous reinoculation were not as severe as those produced by the other strains with which these patients had been earlier inoculated.

Certain of the patients received a second heterologous reinoculation, as shown in Table 4.

The severity of infections subsequent to the second heterologous reinoculation is for the most part closely similar to that exhibited following the first reinoculation, which is more clearly developed by Table 5.

Table 5—Means and standard errors for data relating to the five colored patients of Table 4.

	Primary inoculation	1st reinoculation	2nd reinoculation
ALL PARASITES			
Days 1st to max.	8.6 ± 0.68	8.4 ± 1.12	6.4 ± 0.81
Mean max. per cmm.	93,230 ± 37,052	41,928 ± 23,893	53,064 ± 28,574
GAMETOCYTES ONLY			
Days 1st to max.	13.4 ± 1.60	13.0 ± 3.36	16.2 ± 4.62
Mean max. per cmm.	3,394 ± 2,030	4,234 ± 1,851	272 ± 236
CLINICAL ACTIVITY			
Days 1st to last	26.6 ± 9.90	9.0 ± 3.91	9.4 ± 4.77
Days actual fever	11.0 ± 1.95	4.6 ± 1.47	4.4 ± 0.81

Two of the colored patients listed above, who had been successively inoculated with the Cuban, Mexican, and Panamanian strains, received a third reinoculation with the Long strain. The resulting experience is summarized in Table 6.

In one of the patients the severity of the infection following the third heterologous reinoculation was greater than that following the first or second; in the other, the duration of clinical activity was similar to that shown in Table 5 for the second heterologous reinoculations.

Conclusions

Convalescents from artificially induced falciparum infections usually exhibit a distinct clinical tolerance when artificially reinoculated with a heterologous strain of this parasite, manifested by a shortened period of clinical activity. The infections observed in white patients thus reinoculated closely resemble the primary infections usually seen in colored patients, both exhibiting but one wave of trophozoites, which is, however, followed by a wave of gametocytes, the appearance of which marks the termination of clinical activity. Therapeutic interference is not so frequently required to control the exuberance of the infection. The density attained by

Table 4.—Salient features of infections resulting from second heterologous reinoculation

Primary	Strain: Reinoc. 1st	Strain: Reinoc. 2nd	Race of Patient	Maximum Density: All: Days from 1st par. to max.	Maximum Density: All: Per cmm.	Maximum Density: Gametocytes: Days from 1st par. to max.	Maximum Density: Gametocytes: Per cmm.	Clinical Activity: Days: 1st to last	Clinical Activity: Days: Actual fever	Clinical Activity: Therapeutic interference
Costa	Harden	Bynum	White (1)	7	50,300	15	40	12	7	0
Cuban	Long	Bynum	White (1)	6	30,500	0	0	8	8	0
Cuban	Mexican	Panamanian	Colored (2)	9	91,200	21	90	6	6	0
Cuban	Mexican	Panamanian	Colored	6	11,700	0	0	2	2	0
Long	Panamanian	Mexican	Colored (1)	6	12,800	15	40	8	5	0
Mexican	Long	Panamanian	Colored (1)	4	2,020	28	10	28	6	0
Panamanian	Costa	Trinidad	Colored (1)	7	147,600	17	1220	3	3	1
All			White	6.5± .50	40,400± 9,900	7.5± 7.5	20± 20	10± 2	7.5± .50	0
			Colored	6.4± .81	53,064± 28,574	16.2± 4.6	272± 236	9.4± 4.8	4.4± .81	0 1
All			7	6.4	49,440	13.7	200	9.6	5.3	1

Table 6.—Salient features of successive infections in patients (colored) receiving three heterologous reinoculations

Patient	Strain Inoculations: Prim.	Strain Inoculations: Reinoc.	Maximum parasite density: All: Days from 1st par.	Maximum parasite density: All: Parasites per cmm	Maximum parasite density: Gametocytes: Days from 1st par.	Maximum parasite density: Gametocytes: Per cmm.	Clinical activity: Days 1st to last	Clinical activity: Days actual fever	Clinical activity: Therapeutic Intf.
B-1840	Cuban		10	225,900	17	10,300	27	12	+
B-1840		Mexican	9	37,000	17	9,400	23	9	0
B-1840		Panamanian	9	91,200	21	90	6	6	0
B-1840		Long	9	23,070	15	3,030	5	5	0
B-1853	Cuban		6	61,800	14	5,780	64	10	+
B-1853		Mexican	6	19,600	13	3,740	6	4	0
B-1853		Panamanian	6	11,700	0	0	2	2	0
B-1853		Long	7	74,340	16	2,250	23	9	0

the parasitemia of the heterologous reinoculation may exceed that of the original infection but, is usually lower. Gametocyte production following the heterologous reinoculation of colored patients is frequently greater than that observed during the primary infection. These circumstances indicate that the immunity developed during convalescence from a falciparum infection has an appreciable heterologous value.

STUDIES ON IMPORTED MALARIAS: 2. ABILITY OF CALIFORNIA ANOPHELINES TO TRANSMIT MALARIAS OF FOREIGN ORIGIN AND OTHER CONSIDERATIONS[1]

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Introduction

This report records observations made on the ability of California anophelines to transmit malarias of foreign origin. A previous report has given in general (Young, Stubbs, Moore, Ehrman, Hardman, Ellis and Burgess, 1945) the results obtained through September 30, 1944, for the several laboratories of the "Imported Malaria Studies" program. The present report will deal more in detail with the results of experiments conducted in the laboratory which was located in the Letterman General Hospital and which operated from November, 1943, to December, 1944.

Methods

The usual procedure was to feed mosquitoes upon volunteer returned service men relapsing with foreign malaria. In some instances mosquitoes so infected were applied to mental patients requiring malaria therapeutically at State Hospitals. From such induced cases other feedings were made to test further the infectivity of particular infections.

At the time of application of mosquitoes, white blood cell counts were usually made, as well as routine blood and exflagellation smears.

When the work first began, an attempt was made to select patients with demonstrable gametocytes. Later this became impractical and some patients were tested without determining the gametocyte density beforehand.

After feeding, the mosquitoes were placed in a constant temperature and humidity cabinet controlled to 75°F. plus or minus 1.0° and a relative humidity of 80-90 percent, with the exception of the first few weeks during which time the usual temperature maintained was around 73° F.

[1]Contribution from the Imported Malaria Studies program of the Office of Malaria Investigations, National Institute of Health, and the office of Malaria Control in War Areas.

[2]The following hospitals made available soldiers with relapsing malaria infections: Letterman General, Hammond General, Dibble General, Oakland Naval, and U. S. Marine. The mental hospitals cooperating were the California State Hospitals at Napa and Agnew and the U. S. Veterans Hospital at Palo Alto. To these, and especially to Letterman General Hospital which also furnished laboratory quarters, we express appreciation. Also, thanks are due to the Division of Entomology of the University of California and to the California project of Malaria Control in War Areas and particularly to G. E. Washburn and R. Rosensteil, for their valuable cooperation.

While in the cabinet the mosquitoes were fed nightly by placing on top of the jars gauze strips wet with 5 percent glucose solution. These strips were removed each morning and washed. The mosquitoes were dissected 6, 8, 10, 12 and 14 days after feeding and on such other days as indicated.

Four species of California mosquitoes were used in this study, viz., *Anopheles maculipennis freeborni* Aitken, *A. m. occidentalis* (D and K), *A. psuedopunctipennis franciscanus* (McCracken) and *A. punctipennis* (Say). *A. m. freeborni* was used as the standard testing species and the others were compared with it. For the purpose of this report, the nomenclature of Aitken (1945) has been followed.

The origins of the different species used were as follows: *A. m. freeborni*—Marysville, Auburn, Riverside, and Merced, California; *A. punctipennis*—San Francisco Bay Area and Auburn, California; *A. p. franciscanus*—San Francisco Bay Area and Marysville, California; *A. m. occidentalis*—San Francisco Bay Area, California, and adjacent coastal region.

A colony of *A. m. freeborni* was established from field caught females which came principally from the vicinity of Marysville, California. The mosquitoes used for transmission came from the colony, except a few which were collected in the field for special purposes.

A "lot" of mosquitoes is defined as a group of mosquitoes of the same species fed upon a patient at one time. It usually consisted of 100 or more mosquitoes. A lot was considered infected which showed one or more mosquitoes with oocysts on the gut, sporozoites in the glands, or both.

Starting at 501, each infection was given a serial number. Symbols were designated for the various origins as follows: N. G.—New Guinea; G—Guadalcanal; N. B.—New Britain; N. Ge.—New Georgia; B—Bougainville; Si—Sicily. The origin symbol follows the serial number.

During the course of this work, about 100,000 anophelines were handled. Of these, 89,497 were reared in the insectary as follows: *A. m. freeborni* 84,668; *A. punctipennis* 2,124; *A. m. occidentalis* 2,175; and *A. p. franciscanus* 530. The rest were field caught specimens.

A total of 89 feedings (lots) was made in which 11,592 (79 percent) mosquitoes fed out of 14,640 applied. Seventy-three patients (relapsing soldiers—64; neurosyphilitics—9) who had foreign malarias, were fed upon.

Observations

Origin and Infectivity of the Relapsing Malarias. Sixty-four lots of mosquitoes (*A. m. freeborni*) were fed upon 63 returned service men relapsing with foreign *Plasmodium vivax* malaria, as shown

in Table 1.

Table 1.—Infectivity of foreign relapsing *P. vivax* to *A. m. freeborni*

Probable Origin of Infection	Patients upon whom mosquitoes were fed		Mosquitoes fed on patients	
	Number	Number Who Infected Mosquitoes	Number of Lots Fed	Number of Lots Which Became Infected
Guadalcanal	26	24	26	24
New Guinea	18	10	19	10
Other South Pacific*	18	14	19	14
Total South Pacific	62	48	64	48
Mediterranean	1	0	1	0

* These include New Georgia, New Britain, Australia, Bougainville, and Indefinite.

The infectivity of the *vivax* infections to *A. m. freeborni* is shown in Table 2.

Table 2.—Feeding, dissection and infection data in 48 lots of A. m. *freeborni* infected with *P. vivax*

Mosquitoes Fed	7,444
Mosquitoes Dissected	2,711
Mosquitoes Infected	1,428
Per Cent Infected	52.67

The 17 lots which failed to show infections contained 2,180 mosquitoes of which 660 were dissected.

The average rate of infection for all of the mosquitoes in the total 65 lots was 42.36 per cent.

The infection rates of the forty-four infections originating from Guadalcanal and New Guinea were compared. Out of the 1,449 mosquitoes dissected which had fed upon Guadalcanal infections, 41 per cent were infected; of the 831 of those fed upon New Guinea infections, 37 per cent were infected. It appears that there is little diffence in the infectivity of the infections originating from these two areas.

In addition to the above *P. vivax* infections, there was one *P. falciparum* infection tested which apparantly originated from Guadalcanal. It showed 0.8 gametocytes per 100 white blood cells (86 per cmm.) at the time of feeding, at which time exflagellation was demonstrated.

Twenty-eight mosquitoes were dissected. One gut with two oocysts was found on the 8th day of incubation. Gland sporozoites were not found. This infection occurred even after the patient had started receiving treatment for his malaria.

Actually it is believed that a higher proportion of mosquitoes became infected than appears in the above figures because when dissections were made the mosquitoes which had not taken a blood meal and which had survived were dissected along with those which had taken blood meals. Therefore, the infectivity as expressed represents the minimum infectivity.

The fact that 74 per cent of the lots of mosquitoes fed upon the soldiers relapsing with *P. vivax* showed some infections and that 42 per cent of all mosquitoes applied became infected, indicates that under the conditions of the experiments, these foreign malarias readily infected *A. m. freeborni.*

With two exceptions, each soldier was tested only one time. It is likely that a relapsing soldier might not be infective to mosquitoes at one time and might be infective at another time in the relapse. So, some of those which did not infect mosquitoes might have done so at another trial.

Apparently it can be concluded that these relapsing foreign *vivax* malarias show a high rate of infectivity to *A. m. freeborni.*

The Infectivity to A. m. freeborni of Seven Foreign vivax Malarias Induced in Nine Neurosyphilitics Compared to the Same Infections in the Relapsing Soldiers. Further tests of the infectivity of these foreign malaries were made by using neurosyphilitics to whom these malaries had been transmitted. This infectivity is compared to that of the same strains in the soldiers.

The malaria induced in the neurosyphilitics was in the primary attack at the time of feeding mosquitoes. In the soldiers, infection 528 apparently was in the primary attack and the rest were relapses.

Table 3 shows the results of these experiments.

Table 3—Infectivity to *A. M. freeborni* of seven foreign *vivax* malarias inducted in nine neurosyphilitics compared to the same infections in relapsing soldiers.

	Neurosyphilitics			Relapsing Soldiers		
Infection Number	Gametocytes per 100 wbc	Mosquitoes Dissected	% Infected	Gametocytes per 100 wbc	Mosquitoes Dissected	% Infected
506G	10	71	94	6	69	93
528NG	2	23	87	2	103	89
541G	12	27	100	3	123	76
541G	2	18	72			
543G	16	24	88	2	66	65
543G	10	19	0			
552NG	4	36	83	3	152	24
559NG	4	32	81	8	40	88
561NG	7	24	96	13	50	74
Totals and Averages	7.4	274	82.9	5.3	603	66.3

All of the feedings on the neurosyphilitics were made during the primary attack when the average gamtocyte count was 40 percent higher than in the relapses of the soldiers.

There were 25 percent more infected mosquitoes in the feedings upon the neurosyphilitics than in those of the relapsing soldiers. However, with the exception of 552-NG in the soldier patient, all of the trials gave relatively high infection rates in both soldiers and neurosyphilitics.

Infectivity of Foreign vivax Malarias to Various Species of California Mosquitoes. As far as could be determined there are few experimental data concerning the ability of western anophelines to act as vectors for malaria and apparently there are no data on California mosquitoes. To obtain such information, experiments were run to compare *A. p. franciscanus, A. m. occidentalis,* and *A. punctipennis* to the standard testing species of *A. m. freeborni.*

The results of feeding these different species of mosquitoes simultaenously on foreign malarias are shown in Table 4.

All four species became infected. In every case where the *A. m. freeborni* became infected, the other species tried at the same time also became infected. When *A. m. freeborni* did not become infected, neither did the other species.

Inasmuch as each of the species differed in the proportion of those applied that bit, a comparison of the percentages of infection might not be representative of the susceptibility of that species to malaria. A comparison of the number of oocysts on the gut might be a better measure.

Such a comparison (Table 4) showed that the different species involved in a single feeding had about the same intensity of gut infection. Furthermore, sporozoites were found in all of the species tried.

A single attempt was made to transmit the infection to a neurosyphilitic by each *A. m. occidentalis* and *A. punctipennis.* Both resulted in infections. No attempt to transmit was made with *A. p. franciscanus.*

Length of Sporogonous Cycle of Foreign P. vivax in A. m. freeborni. Out of 57 lots of mosquitoes infected upon relapsing soldiers and neurosyphilitics, 29 were followed by daily dissections to obtain the length of the sporogonous cycle in *A. m. freeborni* by determining the first day of appearance of sporozoites in the glands. These data are presented in Table 5.

Table 5.—Length of sporogonous cycle of foreign *P. vivax* in *A. m. freeborni*

Donor	Incubation Temperatures	First Day of Sporozoites In Glands in 29 Lots of Mosquitoes						Total Lots	Average Days
		9	10	11	12	13	14		
Soldiers	75° F.	5	8	4	2	0	0	19	10.16
Soldiers	73° F.	2	3	0	0	0	1	6	10.33
Neurosyphilitics	75° F.	1	3	0	0	0	0	4	9.75
TOTAL		8	14	4	2	0	1	29	10.07

Table 4.—Comparison of infectivity of foreign *vivax* malaria to various species of California mosquitoes

Infections	A. m. freeborni			A. punctipennis			A. m. occidentalis			A.p. franciscanus		
	Diss.	Inf.	Intensity oocysts	Diss.	Inf.	Intensity oocysts	Diss.	Inf.	Intensity oocysts	Diss.	Inf.	Intensity oocysts
503G	44	18	2.2	31	4	1.5						
541G*	18	13	1.0	19	8	1.0						
543G*	24	21	3.2	24	14T	3.8						
543G*	19	0	0.0	15	0	0.0	8	0	0.0			
552NG*	36	30	1.1							9	4	1.0
560NG	74	48	1.6							14	8	1.3
559NG*	32	26	1.6				24	19	1.1			
561NG*	24	23	2.7				27	17T	2.8			

Legend:
In determining the average intensity of the gut infections the number of oocysts on the guts were expressed as follows: 1–9 oocysts = +; 10–24= ++; 25–99 = +++; 100+ = ++++. In the above table these intensity groups were averaged and expressed in numbers, i.e., the average of + and ++ is expressed as 1.5
* – Indicates feeding made in neurosyphilitics in whom the foreign malarias had been induced. The other feedings were on the relapsing returned soldiers.
T – Transmission to another patient tried and successful.

The above table indicates a rather rapid development of the infection in the *A. m. freeborni.* Fourteen other infected lots of this species dissected at 2 day intervals were not included in the above table but the length of the sporogonous cycle appears to be similar.

The shortest sporogonous cycle noted was from Infection 506-G which had been transmitted to a neurosyphilitic. Sporozoites were found in the glands on the 8th day after incubation at 75° F. This was not included in the above table as dissections had not been done daily.

Three experiments were run comparing *A. m. occidentalis, A. p. francisanus,* and *A. punctipennis* with *A. m. freeborni* as to length of sporogonous cycle. In each instance the two species involved were fed on the patient at the same time and kept under identical conditions at 75° F.

In the two experiments testing *A. punctipennis* and *A. m. occidentalis* against *A. m. freeborni,* all showed gland sporozoites on the tenth day. In the third experiment, *A. p. franciscanus* showed gland sporozoites on the tenth day. However, the control *A. m. freeborni* were not dissected on the tenth day but dissection on the ninth day had shown nearly mature oocysts. This, in addition to the average findings of the cycle (Table 5), indicates that the development in *A. m. freeborni* is 10 days and that sporozoites probably would have been found in the glands on that day had dissections been done.

These results indicate that the length of the sporogonous cycle is the same (10 days) in the four mosquito species tested. This supports the data in the Table 4 indicating that the susceptibility of these four species to foreign *vivax* is similar.

Transmission of Infections to Man By Mosquitoes. While the presence of normal sporozoites in the glands is presumptive proof of transmissibility of malaria, certain lots of infected mosquitoes were applied to neurosyphilitics for final proof.

The data on the attempts are shown in Table 6. Also included in this table are the attempts to infect mosquitoes from the induced cases.

As seen in Table 6, attempts were made to transmit by mosquitoes 19 infections to 23 persons in 24 trials. Seventeen different strains were transmitted successfully to 21 patients in 22 attempts.

Twelve infections were successfully transmitted by mosquitoes to each of 12 patients, and 5 infections transmitted to 2 patients each. One patient (R. P.) became infected with both 506G and 541G . One patient (W. M.) was infected by blood transfer.

Table 6.—Attempts to transmit foreign *vivax* malaria to man by mosquitoes

Transmission Attempts	Strain No.	Patient Name	Race	Days Pp.	Days Inc.	Resulting Infections in Man: Mosquitoes Subsequently Fed Species	Infected	Remarks
1	506G	M. V.	N	14	12	F	+	
2	506G	R. P.	W	14	13			
3	510NB	A. P.	W	15	15			
4	513G	H. G.	N	11	14			
5	516G	T. C.	C	13	13			
6	518NG	F. R.	N					Had Malaria Before
7	519B	L. D.	N					Had Malaria Before
8	520NG	R. H.	W	12	16			
9	523G	W. M.	W	12	10			
10	524G	J. Z.	W	12	10			
11	528NG	T. B.	W	13	12			
12	528NG	G. W.	W	13	12	F	+	
13	534NG	W. K.	W	11	11			
14	535NG	V. S.	W	16	13			
15	541G	R. F. B.	W	10	10	F	+	Transmitted to R. P.
16	541	R. .P.	W	16	16	F, P	+,+	
17	542G	H. B.	W	29	19			
18	543G	J. M.	W	13	14			Blood to W. M.
	543	W. M.	W	Blood Transfer from J.M.		F. P.	+,+	Transmitted to P. K.
19	543	P. K.	W	19	11	F, P, O,	—,—,—	
20	552NG	J. P.	W	12	14	F, P_F	+,+	
21	559NG	G. R.	W	12	12	F, O	+,+	
22	560NG	E. P.	W	20	18			
23	561NG	E. R.	W	10	11	F, O	+,+	Transmitted to S. L.
24	561	S. L.	W	12	11			
Totals 24	19	23		309	287	9	8	
Averages				14.05	13.05			

Legend: F = *A. m. freeborni*; P = *A. punctipennis*; O = *A. m. occidentalis*; P_F = *A. p. franciscanus*; N = Negro; W = White; C = Chinese; — = No infection + = Infection; Pp = Prepatent period of parasites; Inc = Incubation period of symptoms.
Transmission of all by *A. m. freeborni* except attempt # 24 which was by *A. m. occidentalis* and # 19 by *A. punctipennis*.

Two transmission attempts failed. The recipients were Negroes who had had malaria therapeutically before. It is not possible to state whether either the race of the patient, the previous malaria, or both, operated against the infection developing. It is interesting that the other two Negros involved (M. V. and H. G.) developed malaria producing at least 16 and 15 paroxysms respectively. In these two cases, the race seemed to exert no influence.

On the other hand, one white patient (R. P.) developed infections after each of two transmission attempts (506G and 541G). With the first infection (506G) he had 12 paroxysms. He was given the second malaria (541G) five months after the first one. The second infection was not followed long enough to determine the number of paroxysms produced. While it is not known how much immunity the first infection exerted against the second infection in the length of the clinical manifestations of the latter, it is seen that the immunity was not great enough to prevent the second infection from developing within the normal range (16 days).

Development of P. vivax Infections in A. m. freeborni at Outside Temperatures. Experiments were set up to determine how these foreign malarias would develop at outdoor temperatures. Both field caught and laboratory-bred *A. m. freeborni* were employed. In each experiment, one-half of the mosquitoes which had been applied to the malarious patient were put into a constant temperature cabinet maintained at 75° F. in the San Francisco laboratory. The rest of the mosquitoes were put under shelters at outdoor temperature. They were handled in the routine manner otherwise.

Temperature readings were taken from the official weather reports. The San Francisco readings were taken at the weather bureau several miles away from the barn where mosquitoes were kept. Sacramento readings (nearest weather station to Marysville) were taken about 50 miles from Marysville but were in the same general climate. Readings taken at Marysville during this time were generally the same as shown by the weather bureau records at Sacramento.

The temperature readings at San Francisco and at Sacramento are shown in Table 7.

Table 7.—Temperature readings during outdoor experiments

	Sacramento, Calif.		San Francisco	
Temperatures ° F.	9/26 - 10/11	10/4 - 10/19	9/20 - 10/5	10/2 - 10/21
Maximum Range	99 -72	86 -72	74 - 60	76 - 58
Maximum Average	81	78	68	66
Minimum Range	59 -51	54 -50	58 - 50	57 - 50
Minimum Average	53	52	53	53
Mean Range	80 -62	70 -62	64 - 56	64 - 54
Mean Average	67	65	61	59

The first experiment was with part of the mosquitoes being kept under a porch in Maryville. This experiment was started on September 26th and ended October 11, 1944. The strain used was 552-NG which had been induced in a neurosyphilitic.

Of those kept entirely in Marysville, 12 out of the 16 mosquitoes showed infections whereas out of those kept in the laboratory 30 out of 36 were infected.

The mosquitoes kept in the laboratory at 75° F. showed gland sporozoites on the 11th day. At Marysville, infections developed more slowly. Oocysts on the ninth day were about the same size as the fifth day oocysts incubated at 75° F. The longest kept at Marysville were for 15 days at which time they showed oocysts ranging from half grown to almost mature. The number of the oöcysts was about the same under each condition. Some of the mosquitoes were kept at Marysville for different intervals and then brought into the laboratory. These developed oocysts and gland sporozoites but the appearance of the latter was delayed considerably over those kept entirely in the laboratory at 75° F.

A second experiment was run starting October 4, 1944, at Marysville under the same conditions using Infection 541-G, which had been induced in a neurosyphilitic. Mosquitoes dissected on the 7th day showed no infection while oocysts were found on the fifth and sixth day in those kept at 75° F. On the seventh day, some mosquitoes kept outside at Marysville were put at 75° F. and on the ninth day these showed early oocysts. Others kept at Marysville and dissected on the fifteenth day showed only young oocysts about the size of the 6-7 day oocysts kept at 75° F. The controls kept at 75° F. showed gland sporozoites on the 10th day.

These results indicate that infections can occur in mosquitoes in the vicinity of Marysville under the conditions experienced, but develop more slowly than those kept at 75° F. It is to be expected that gland sporozoites would have developed if the mosquitoes had been left there long enough. During the warmer seasons it is to be expected that the infections would mature faster.

Two experiments were run at San Francisco with one half of the mosquitoes kept in a barn in the city and the others at 75° F. in the laboratory.

The first of these was with 564-NG starting September 20, 1944. Between the fifth and 15th day of development dissections were made. No infections were found in 38 dissections of the mosquitoes kept in the barn; of those kept at 75° F. 22 were dissected and all were infected. In the latter the infection was heavier than average and sporozoites were found in the glands on the ninth day.

The question then arose whether the infections were sterilized or latent in the mosquitoes kept in the barn. To elucidate this point

another experiment was run in which mosquitoes were placed in the barn for varying lengths of time and then put at 75° F. The results are shown in Table 8.

Table 8.—The development of *P. vivax* infections in *A. m. freeborni* under different conditions of temperature in San Francisco (Oct. 2, 1944). Strain 559.

Days Kept		Mosquitoes		
In Barn	Then At 75° F.	Dissected	Infected	Remarks
7	0	8	0	
8	0	8	0	
9	0	4	0	
11	0	7	0	
12	0	11	0	
14	0	7	0	
17	0	3	0	
5	2	4	0	
5	4	3	3	Small early oocysts
7	10	3	2	Sporoluating oocysts
9	2	8	5	Small early oocysts
19	7	4	3	Oocysts almost mature
0	12	32	26	Controls — developed gland sporozoites in 11 days

Five other mosquitoes which were incubated at 75° F. for 5 days and then put in the barn showed very little progression in development of the infection 11 and 12 days later.

Another experiment was run beginning October 6th using 561—NG in which the glands had sporozoites on the 10th day at 75° F. Some of these mosquitoes were removed after 48 hours from the 75° F. temperature and placed in the barn. The oocysts showed little development after being in the barn for as long as 8 days.

These experiments were run during what is ordinarily the warmest part of the year in San Francisco. According to the comparative data of the San Francisco weather bureau, September has been the warmest month for a 70 year period, with an average of 61.5° F. and October was the next warmest with 60.8° F.

From the above results, it seems evident that under the conditions of the experiment, infections would not develop to maturity in mosquitoes at the San Francisco temperatures.

Thus, even if mosquito carriers were present in the city an outbreak of malaria would not be expected if mosquitoes rested outdoors.

Temperature ranges shown above did not sterilize the mosquitoes even when they were kept outside for as long as 19 days. The infections subsequently developed when the mosquitoes were taken to 75° F. Thus, there is a chance of the mosquitoes biting a person and resting in a warm place, such as a house, and developing infections. However, this would not be expected ordinarily.

Furthermore, there appear to be few, if any, *A. m. freeborni* in the city of San Francisco.

So, the possibility of outbreaks of malaria in San Francisco seem very remote and would occur only under unusual conditions and probably would not be widespread.

Relationship of P. vivax Gametocytes to Infection in A. m. freeborni. In all cases, gametocytes were counted and expressed as the number per 100 white blood cells. In 31 instances, white blood cell counts were made at the time of feeding the mosquitoes and in these the number of gametocytes per cmm. of blood was calculated also. When a graph was plotted showing the percentage of infection in the mosquitoes against the number of gametocytes per 100 w.b.c. and against the number of gametocytes per cmm. of blood, it was found that the curves of these two methods of counting correlated closely. The white blood cell counts in 31 soldiers averaged about 6000 per cmm.

The relationship of the mosquitoes infected to the number of gametocytes is presented in Table 9.

Table 9.—Relationship of *P. vivax* gametocytes per 100 wbc to *A. m.* freeborni infected in 65 feedings of relapsing foreign *P. vivax*.

Gametocytes per 100 WBC	Mosquito Lots		Mosquitoes		
	Fed	Infected	Dissected	Infected	% Infected
Less than 1	9	2	419	41	9.8
1 — 5 *	41	33	2082	859	41.3
6 — 10 *	7	7	339	250	73.8
11 — 15 *	7	6	484	278	57.5
32	1	0	47	0	0.0
Averages & Totals 4.5	65	48	3371	1428	42.36

* In the three groups including the range of gametocytes from 1 to 15 per 100 white blood cells, the average infection of the mosquitoes was 47.8 percent.

It is apparent from the data that a sharp rise in infections came when one or more than one gametocytes per 100 w. b. c. (60 per cmm.) were present. The most infective range (73.8 percent) was when from 6 to 10 were present.

The highest gametocyte count encountered was in 545-NG which showed 32 gametocytes per 100 w.b.c. (1440 per cmm). The proportion of males to females was about 1:10 or less. However, this discrepancy in sexes does not explain why it did not infect, as there were still enough males to produce a good infection as shown by other cases. Fourteen days later mosquitos were fed on the same patient with a gametocyte count of 5 per 100 w.b.c. (4 females, 1 male). No infections were found out of 40 dissections.

No reason for such a high gametocyte count failing to infect can be offered.

Conversely, two lots of mosquitoes became infected when fed on patients (558-G and 552-NG) showing no gametocytes per 100 w.b.c. One of these (552-NG) showed 30 percent of 103 mosquitoes infected; the other (558-G) showed 8 percent of 66 mosquitoes infected.

Oddly enough, 552-NG had not produced infections in mosquitoes 14 days earlier with 5 gametocytes per 100 w.b.c. (3 males, 2 females).

From the above, it appears that the following conclusion can be drawn. Foreign relapsing *P. vivax* is not always infective to *A. m. freeborni* in direct proportion to the number of gametocytes present. Generally, however, infections are most likely to be produced when there are between 1 and 15 gametocytes per 100 w.b.c. (60-900 per cmm.) with the highest infectivity (73.8 percent) within the range of 6-10 gametocytes per 100 w.b.c. (360-600 per cmm.).

Relationship of Exflagellation of P. vivax Gametocytes to Infection in the Mosquito. Exflagellation smears were made at the time of feeding as an aid in demonstrating the maturity of the male gametocytes. In general the method used was similar to Shute's technique as described by James (1934), except that slides remained in the moist chamber usually for 5, 10, 15, and 20 minute periods and some for 25 minutes. Both partial and complete exflagellation were recorded as exflagellation. The relationship of exflagelation to infection in the mosquito in 59 trials is set forth in Table 10.

Table 10.—Relationship of exflagellation of *P. vivax* gametocytes to subsequent infection in *A. m. freeborni*.

Exflagellation	Mosquitoes Infected	Number Trials
+	+	22
+	−	4
−	+	24
−	−	9

+ = Positive; − = Negative.

When exflagellation was demonstrated, infection in the mosquitoes usually occurred also (22 out of 26). This is to be expected. However it is further seen that infection occurred in a high proportion of times when no exflagellation was demonstrated (24 out of 33). One suspects that this might be a fault of the technique.

Relationship of Number of Malaria Attacks to Number of Gametocytes Produced and Infection of Lots of Mosquitoes. When mosquitoes were fed upon a relapsing soldier, an effort was made to determine how many attacks of malaria the patient had experienced from that infection. The first attack of malaria regardless of its relationship to suppressive drugs was designated as the primary attack. In many instances, the primary attack had been delayed considerably due to suppressive drugs. The next attack was designated as the first relapse and subsequent relapses were numbered consecutively.

After determining the relationship of gametocytes to infection of the mosquito (Table 9), the question arises about the appearance of gametocytes in the relapses and if present, whether they produce infections in the mosquitoes. Some workers believe that gametocytes become rarer as relapses occur.

The data on 64 cases are shown below in Table 11.

From Table 11 under "Gametocyte Counts", it is evident that gametocytes were produced as long as the relapses occurred. Although the sample of 64 trials was too small to indicate accurately whether fewer gametocytes were produced as relapses progressed, such a trend is not too evident from the above data.

Assuming that the gametocytes produced in the later relapses are as viable as those produced earlier, according to the interpretation of Table 9 it appears that sufficient numbers of gametocytes were produced in the later relapses to infect mosquitoes. Upon examining "Mosquito Feedings" in Table 11, it is seen that mosquitoes were infected even through the 17th relapse (18th attack). The failure of infection on the 20th relapse (21st attack) apparently is not indicative as the gametocytes were rare and also because experience in other laboratories indicates infection in even later relapses.

On the basis of the data given above, it seems reasonable to expect that some of the patients with foreign *P. vivax* will infect mosquitoes as long as relapses occur. The gametocytes appearing in the later relapses appear to be as infective as those produced in the earlier attacks.

Intensity of the P. vivax infections in A. m. freeborni. The number of oocysts per gut were counted or in heavy infections estimated, and recorded under the following intensity groupings: 1 — 9, +; 10 — 24, ++; 25 — 99, +++; 100+, ++++. Sporozoites were grouped as follows: 1 — 9, +; 10 — 99, ++; 100 — 999, +++; 1000+, ++++.

In 33 lots, both gut oocysts and gland sporozoites were found. The distribution of the infected mosquitoes in these lots according to the above groupings is shown in Table 12. The gut infections up to the first day of gland sporozoites and the gland sporozoites on or after the eleventh day were tabulated.

Table 12.—Intensity of infection of foreign *P. vivax* in *A. m. freeborni*

	Number of Specimens Under Each Intensity Grouping				
	+	++	+++	++++	Total
Guts with Oocysts	373	183	101	37	694
Glands with Sporozoites	122	138	127	67	454

Table 11.—Relationship of number of malaria attacks (*P. vivax*) to number of gametocytes and infection of lots of mosquitoes

No. Attacks	P	Relapse Number 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	20	Total
Gametocyte Counts																			
Trials	6	11	6	4	3	3	3	3	5	5	2	3	3	1	1	3	1	1	64
Av. per 100 wbc	3.8	4.6	2.0	11.1	1.3	3.0	8.3	4.0	6.8	4.8	7.5	4.3	2.1	1.0	4.0	4.0	2.0	1.0	4.5
Mosquito Feedings																			
Infected	4	10	5	2	2	3	3	2	3	5	1	2	1	1	1	2	1	0	48
Not Infected	2	1	1	2	1	0	0	1	2	0	1	1	2	0	0	1	0	1	16

P = Primary Infection

Table 13.—**Unusually heavy infection in *A. m. freeborni*** showing intensity of oocysts *P. vivax* of foreign origin

Infection	Donor	Relapse Number	Gametocytes per 100 wbc	cmm.	Guts Dissected	Infected	Range of oocysts per gut and number of guts in each range 1-50	50+	100+	200+	300+	400+	500+	800+
506G	S	7	6	462	33	28	15	1	5	1	4		2	
506G	N	P	10	1029	27	26	4	4	8	3	3		3	1
524G	S	6	11	739	23	23	5	7	8	3				
505G	S	8	9	468	32	25	7	7	5	7	4	1	1	
543G	N	P	16	806	16	13	@	@	10@A					

Dissections made from 5th day up through last day before sporozoites found in glands.
@ Actual oocysts counts not made. Reported as +++ (25-99, or ++++ (100 and above).
S Relapsing soldier.
N Neurosyphilitic in whom infection had been induced.
A Also *A. punctipennis* showed 6 guts with over 100 oocysts.
P Primary attack.

From these data, it is apparent that most (80 per cent) of the infected guts had between 1 and 24 oocysts and that most (85 per cent) of the glands contained between 1 and 100 sporozoites.

In 5 cases, unusually heavy infection were seen. The origin of all of these infections was probably Guadalcanal.

The data on these infections are shown in Table 13.

About 500 oocysts are about as many as we can find reported for any mosquito. Thus, the five infections are noteworthy.

The intensity of sporozoites in the glands was correspondingly high in the above infected mosquitoes.

The gametocyte counts for these five infections averaged 10.5 per 100 w.b.c. as compared to the average of 4.5 per 100 w.b.c. for the 65 total counts made. This increase in gametocytes would not seem to be enough to account for such heavy infections. Gametocyte counts higher than the above did not produce correspondingly heavy infections.

The above data suggest that these particular infections are more infective to mosquitoes than usual. Other infections also apparently originating from Guadalcanal did not show such heavy infections. However, the very fact that such heavy infections were produced indicates that some Guadalcanal infections might be more infective than others.

Mosquito Infection after Therapy. In 4 instances mosquitoes became infected when applied after malaria treatment had begun. Three of these were *P. vivax* and one *P. falciparum.*

Table 14.—Mosquitoes infected after patient had received treatment

Infection Number	Mosquitoes Dissected	Infected	Drug	Amount Given Before Biting
V-557-NG	54	48	Quinine Sulfate	15 grains day before
V-543G	66	43	Quinine Sulfate	45 grains i.v. within 24 hours before feeding
V-507-G	20	4	Atabrine*	0.4 gram 2 hours before
F-512-G	28	1	Quinine Hydrochloride	7.5 grains i.v.,b.i.d./3 days

* - Blood level of 50 gammas at time of feeding
v - *P. vivax*
f - *P. falciparum*

This shows that the foreign malarias can infect mosquitoes after treatment has been started. Such results are similar to experiences in the Columbia, South Carolina, laboratory and to that of others where it had been observed that American malarias may infect mosquitoes following some treatment.

Comparison of the Susceptibility of Field Caught and Laboratory Reared A. m. freeborni. Two experiments were run to determine if field-caught adults and insectory-reared (6th generation) adults showed any differences in susceptibility to these foreign malaries.

In each experiment, one-half each of the field and laboratory mosquitoes were kept at 75° F. and one-half under outside conditions at Marysville such as described above. The malaria infections involved were, No. 559 and No. 561, both from New Guinea, which had been induced in neurosyphilitics.

Of the laboratory-reared mosquitoes, out of 64 dissections 40 showed infections; of the field caught specimens, out of 75 dissections, 30 showed infections.

The results indicated definitely that both the laboratory and field-caught mosquitoes were susceptible to foreign malarias. The data are not sufficient to draw final conclusions as to the relative susceptibility of field-caught against laboratory-reared mosquitoes.

Characteristics of Foreign P. vivax Induced in Neurosyphilitics. Slides were made and temperatures were taken at irregular times on the induced cases due to shortage of ward help at the mental hospitals. However, even with such irregular tests, the incubation and prepatent periods as determined are interesting.

The prepatent periods ranged from 10 to 29 (with only one above 20 days) averaging 14.05 days (Table 6). The incubation periods ranged from 10 to 19 days, averaging 13.05 days.

These averages represent the maximum as it is certain that in some cases had slides been made and temperatures taken daily, the observed periods would have been shortened. Even so, the prepatent and incubation periods are relatively short as compared to some of our indigenous "strains" of malaria. Unpublished data from 35 patients with *P. vivax* (St. Elizabeth strain) indicate that the prepatent period is about 15 days and the incubation period about 16 days.

Most of the induced cases showed a quotidian rather than a tertian fever periodicity. However, the use of thio-bismol when one brood of parasites were one-half grown changed the periodicity of the fevers to tertian. This is similar to the reaction of thio-bismol on the St. Elizabeth strain of *P. vivax* which is widely used in this country for the treatment of neurosyphilis (Young, McLendon and Smarr, 1943).

In the infections showing tertain periodicity of fevers, the fevers did not come at 48-hour intervals but at shorter intervals. This is in conformity to observations previously reported (Young, 1944) which showed that three strains of *P. vivax* studied had a fever periodicity of less than 48 hours.

The fever durations, fever peaks, and parasite densities seem to be in the range of that usually found with St. Elizabeth's strain (Coatney and Young, 1942).

No outstanding differences in the course of the infections were noted.

Discussion

A. m. freeborni. No natural or experimental infection rates have been found for *A. m. freeborni* either for California or Oregon, where for years it has been indicted as the principal vector. Two records were found for New Mexico. Barber, Komp and Hayne (1929) found 0.3 percent of 669 wild caught *A. maculipennis* infected in northern New Mexico. They also experimentally infected them with *P. vivax* but gave no figures except that *A. maculipennis* showed more infections than *A. pseudopunctipennis.* Barber and Forbich (1933) in New Mexico found 1.4 percent out of 868 wild-caught infected. Simmons and Aitken (1942) give these as the only infection rates known for *A. m. freeborni.* There appear to be no data on *P. falciparum* or *P. malariae.*

The susceptibility of *A. m. freeborni* to foreign *vivax* malaria appears to be high. Under laboratory conditions at 75° F. the infections in mosquitoes matured in the relatively short incubation period of 10 days. Under outside conditions in Marysville, the infection developed, but more slowly. Outside in San Francisco, the infections did not develop, but the infections were viable and developed when put in warmer places.

In the past, its association with malaria, its habits of entering houses and biting man, and its prevalence has led to its incrimination as the principal vector in California.

Reeves (1944) gave support to this when he showed by precipitin tests that an average of 3 percent of 473 specimens had fed on man. In one area 7.1 percent had taken human blood meals.

The evidence of its susceptibility reported here supports this incrimination.

A. punctipennis. While experiments infecting *A. punctipennis* with plasmodia have been done at other places, it appears that none have been carried out with California or other West Coast specimens. We found the susceptibility to be quite similar to that of *A. m. freeborni* and that sporozoites were developed in 10 days at 75° F. Transmission to man was successful.

In the southeastern states, *A. punctipennis* usually is not considered as being an important vector. It is known, however, to attack man, to be prevalent, and to enter porches and houses.

In California, there are indications on epidemological grounds that it might be a vector of malaria. Lenert (1924), as quoted by Barber, Komp and Hayne (1927), states that *A. punctipennis* is the malaria carrier of the foothills of the Sierra Nevada in California. Herms (1919), suggests *A. punctipennis* as a carrier in Northern California. Simmons and Aitken (1942) quote Herms as saying that this species may play a part in malaria transmission in the Mother Lode Mining region of the western Sierra Nevada foothills of Cali-

fornia.

The experimental evidence obtained by us indicates that it is susceptible to foreign plasmodia.

A. p. franciscanus. This species is considered to be of little importance as a vector. Herms (1919) considers *A. pseudopunctipennis* as a weak or no carrier on the coast of California. Barber, et al. (1929) in New Mexico found none of 118 wild-caught *A. psuedopunctipennis* infected. They infected them in the laboratory with *P. vivax* but gave no figures except that *A. maculipennis* gave a larger infection percentage. Barber and Forbich (1933) found none out of 263 (wild-caught) infected in New Mexico. No data on *P. falciparum* or *P. malariae* seem to be available.

Simmons and Aitken (1942) refer to the above as the only experimental work. They reserve the opinion as to whether it was *A. pseudopunctipennis* or *A. p. franciscanus.*

It does not attack man readily. Reeves (1944) out of 178 specimens found only 0.6 percent as having fed on man.

Our experimental evidence indicates that it is susceptible to foreign *vivax* malaria, being similar to *A. m. freeborni* in this respect. However, because of its habits it is not xpected to participate to any extent in the spread of these foreign malarias.

A. m. occidentalis. Simmons and Aitken (1942) say there is no experimental evidence of the susceptibility of this species. Neither were the writers able to find any such evidence.

Aitken (1945) believes that it probably plays no part in the transmission of malaria because it occurs in a cool climate.

As to the susceptibility, this report shows it can act as a malaria vector experimentally. As to the climate, recoveries of this species have been made as far south as San Diego on the California coast. Along the southern part of the coast, temperatures are high enough for development of infection in the mosquito. Also summer temperatures in Minnesota and Wisconsin, where this species also occurs, would allow for development of plasmodia in mosquitoes.

It is not supposed to be a house invader. Simmons and Aitken (1942) state that it will feed on man readily in the laboratory.

This was also our experience.

Its prevalence and habits, rather than a lack of susceptibility to plasmodia, appear to be the main reasons why it would not be considered as an efficient vector of foreign malarias. The same conclusion can be applied to its relationship to native malarias.

Significance of California Anopheles in Spreading Foreign vivax Malaria Relapsing in Returned Carriers. From the above, *A. p. franciscanus* and *A. m. occidentalis* would not be expected to act as important vectors of imported foreign malaries, even though they are susceptible to the plasmodia.

It is to be expected that *A. m. freeborni* will transmit foreign malarias wherever this mosquito has access to sufficient parasite carriers.

The importance of *A. punctipennis* as a vector is not settled There seems to be reason to expect it to be an efficient vector where it is prevalent enough and malaria carries are available.

It is to be expected that some troops relapsing with foreign malaria eventually will be in areas in California and elsewhere where the above vectors are present. The amount of malaria resulting from such carriers will depend largely upon the control of the mosquito vectors, as no way has yet been found to sterilize the human host of this malaria. With adequate control measures, such outbreaks should be quite limited in number and circumscribed.

Other Parasitological Considerations. Infections passed through the mosquitoes to neurosyphilitics were again transmissible through these mosquitoes. There was no indication that the infections were changed by transmission through these insect hosts.

The only *P. falciparum* encountered showed a light infection upon the gut and no gland infection. There is no evidence that *P. falciparum* from foreign areas will fail to infect *A. m. freeborni*. As few cases of *P. falciparum* are expected to be imported by troops, this particular hazard will be not nearly so great as with *P. vivax*. Further observations are being made, however, upon the susceptibility of *A. m. freeborni* to *P. falciparum*.

The outside experiment in San Francisco raised an interesting point concerning the length of time mosquitoes can retain infections. Mosquitoes kept outside did not show development of oocysts for nearly three weeks but upon being put at a warmer temperature, gut infections began to develop. How long can infections in mosquitoes remain viable at cool temperatures in nature? The answer to this question might have a direct relationship to the malaria picture in California.

Summary and Conclusions

1. Mosquito feedings were made on 64 relapsing soldiers and 9 neurosyphilitics who had foreign *Plasmodium vivax* malaria. 89 feedings were made in which 11, 592 mosquitoes (79 per cent) fed out of the 14,640 applied. 63 of the infections were *P. vivax* and one *P. falciparum*. 62 of the *vivax* infections originated from the South Pacific area and one from the Mediterranean area.

2. Of the 64 mosquito lots fed on 62 relapsing *vivax* soldiers from the South Pacific, 48 became infected. In these 48 infected lots, 2,711 *A. maculipennis freeborni* were dissected showing 1,428 (52.67 per cent) infected. This indicates that foreign relapsing *vivax* malaria readily infects *A. m. freeborni*.

3. Attempts were made to transmit by mosquitoes 19 relapsing infections to 23 patients. Seventeen different infections were transmitted to 21 patients. The two failures were Negroes who had had malaria before.

4. With seven different infections, the relapsing soldiers and neurosyphilitics in the induced primary attack showed relatively high infectivity rates to *A. m. freeborni.*

5. A comparison of the infectivity of foreign malarias to four species of California mosquitoes (*A. m. freeborni, A. punctipennis, A. maculipennis occidentalis* and *A. pseudopunctipennis franciscanus*) was made. All appeared to have about the same susceptibility. All developed sporozoites. Transmission was tried and successful with *A. m. freeborni, A. punctipennis,* and *A. m. occidentalis.*

6. The length of the sporogonous cycle in the above four species at 75 ° F. was about 10 days. The shortest cycle observed was 8 days in *A. m. freeborni.*

7. Infected mosquitoes were kept at outside temperatures during the last of September and the middle of October. At Marysville, California, the infections developed but much slower than those kept at 75° F. In San Francisco the infections did not develop over a 19-day period: however when put at 75° F. these infections developed showing they were still viable.

Thus one could expect foreign malarias to be spread in climate areas similar to Marysville, particularly in the warmer months, but not in San Francisco, as the observations in the latter were made in what is usually the warmest part of the year there.

8. The gametocyte count in relapsing soldiers averaged 4.5 per 100 w.b.c., ranging from 0 to 32 (0 to 1440 per cmm.).

Two lots of mosquitoes were infected when no gametocytes per 100 w.b.c. were seen. Conversely, a very high gametocyte count of 32 per 100 w.b.c. (1440 per cmm.) did not infect.

Foreign relapsing *vivax* was not always infective to *A. m. freeborni* in direct proportion to the number of gametocytes present. Infections occurred most frequently when there were between 1 and 15 gametocytes per 100 w.b.c (60-900 per cmm.) with the highest infectivity (73.8 per cent of the mosquitoes) within the range of 6-10 gametocytes per 100 w.b.c (360-600 per cmm.).

9. Infection in the mosquitoes usually occurred when exflagellation was demonstrated but also occurred frequently when no exflagellation was seen.

10. In general, gametocytes were produced as long as the relapses occurred. Mosquitoes were infected on patients with the 17th relapse (18th attack). It seems reasonable to expect that some of the patients with foreign *P. vivax* will produce gametocytes and will infect mosquitoes as long as relapses occur.

11. The usual number of oocysts upon the guts of infected *A. m. freeborni* was between 1 and 24. The sporozoites in the glands were usually between 10 and 100. In five cases, all from Guadalcanal, unusually heavy infections were seen showing guts with several hundred oocysts. One gut had approximately 800 oocysts. In these heavy gut infections, the gland infections were corresponding high.

12. In 4 instances, *A. m. freeborni* became infected when applied after malaria therapy with quinine or atabrine had begun. Three of these were *P. vivax* and one *P. falciparum*.

13. The foreign *vivax* malarias were infective to the field-caught as well as to the laboratory-reared *A. m. freeborni*.

14. Foreign *P. vivax* induced in neurosyphilitics produced infections similar to those produced by strains of *vivax* in this country, in particular reference to duration of fevers, fever peaks, parasite densities, and response to thio-bismol. The periodicity of the fevers, in those occurring every other day, was less than 48 hours.

15. On the basis of the evidence presented, it appears justified to conclude that control measures are necessary for the foreign malarias brought to the American west coast by relapsing carriers.

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BOOK REVIEW

Studies of Malaria In Cartagena

Thesis for title of Doctor of Medicine and Surgery by
Alvara Ujueta Herrera, Editorial Cromos, Bogota, 1945

This thesis describes a series of medical and entomological surveys undertaken in 1943 and 1944 by the author in Cartegena, Colombia for the purpose of determining the malaria problem and indicating necessary control measures.

Tables are provided to show the specific malaria mortality in comparison with total mortality; the distribution of positive blood slides and spleens by age groups and by City Wards; the seasonal variation of malaria according to parasitic and splenic findings; the correlation between parasitic and splenic findings; the seasonal rainfall and prevailing winds; the seasonal densities of mosquitoes by species; the mosquitoes trapped in various zones of the city; and the correlation between rainfall, mosquito counts and specific malaria mortality.

All of the actual and potential breeding places in the city have been listed. A pertinent fact is the note that a great majority of the mosquitoes found in houses was *A. albimanus* and that only about 1 percent were *A. triannulatus* and none was *A. neomaculipalpus*. Control efforts, however, could not follow strict species sanitation but would have to be generally directed at all three species because they breed in the same places.

The author has shown clearly the existence of a serious malaria problem, its predominance in certain sections of the city, the vector responsible, the location of all breeding places, the creation of temporary breeding places during the rainy seasons, the seasonal variation, and the correlations between rainfall, mosquito densities, and malaria.

With the data provided it would be possible for an operations officer to set up a permanent malaria control program which should be very effective. Certainly with parasitic indices as high as 85 per cent and splenic indices as high is 70 per cent and with the predominance of slides positive for *P. falciparum*, an efficient control. program is a dire necessity.

Vernon B. Link.

THE USE OF DARKFIELD ILLUMINATION IN STUDIES OF MALARIA PARASITES

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A review of the Tropical Diseases Bulletin and the Cumulative Index Medicus for the past 25 years reveals only two titles of papers describing the use of darkfield illumination in studies on malaria parasites (Anderson and Cowdry, 1928; Wolter, 1932). This fact together with the known usefulness of darkfield technique in other fields and the current intense interest in malaria, seemed to justify a reappraisal of the practical value of darkfield illumination in malaria studies. This method has been studied with reference to its practical value in the diagnosis of malaria, in studies on exflagellation, and in observations of the action of antimalarial drugs on the morphology of malaria parasites. The following brief report of this work describes the technique employed and the application of the method on a malaria therapy service for neuro-syphilis.

Apparatus and Technique

Two types of microscopes have been employed. Originally, all observations were made with a special darkfield microscope (Spencer Model 32), using a single oil immersion objective. This was subsequently replaced by a standard triple objective microscope in which the condensor had been replaced by a cardioid type of darkfield condensor, with suitable funnel stops inserted in the 1.9 and 4 mm. objectives, to reduce their apertures. The latter objective (high dry) permitted observation of a larger microscopic field, a decided advantage in exflagellation studies. With this microscope it was also possible to obtain stronger illumination than with the built-in illumination of the special darkfield microscope. Stronger illumination is of particular value in microphotographic work, and for this purpose a lamp (Spencer Model 370) containing a 200 watt projection lamp was employed.

The first step in using such a microscope is to center the condensor, using low power magnification and focusing upon the etched circle on the upper surface of the condensor. This is essential for proper illumination. In selecting slides for use with this technique, it was found that the best results were obtained with those having a thickness of 1.15 to 1.25 mm. When wet preparations were employed, the coverglasses used were either the square or oblong type, the latter proving more satisfactory when large drops of diluted blood were employed. Scrupulous cleanliness of both slides and coverglasses were found to essential for satisfactory results.

To this end, coverglasses were kept in alcohol or acetone containers, and dried with a piece of lint-free cloth before using. Slides were wiped free of dust particles in a similar manner before use. Further details of technique will be mentioned below in connection with the specific procedures for which this technique was employed. The study of wet preparations affords particular interest in that it permits observation of the active, living parasite, undistorted by the application of fixing and staining agents. Properly prepared wet films of whole blood, or blood diluted with citrate, saline, or distilled water can be observed for hours under the microscope.

The use of whole blood has the disadvantage of tending to produce rouleaux and agglutinated masses of red blood cells in which the details of plasmodial morphology are difficult to observe. To some extent this can be overcome by using a small drop of blood and pressing firmly upon the coverglass, sufficiently to obtain a single layer of red blood cells. The use of heparin as an anticoagulant was found to be unsatisfactory due to the tendency to rouleaux formation. The addition of isotonic saline, or of 2 percent sodium citrate, in equivalent amount to the drop of blood often produced undesirable crenation of red cells. The best results were obtained by the addition of a small drop of distilled water, or even ordinary tap water, to the drop of blood. For purposes of simplicity the latter was used routinely, a small drop being transferred upon an applicator stick from the water-faucet to the coverglass.* A small drop of blood from the patient's finger was then placed upon the slide, and the coverglass applied so that the drop of water was superimposed upon the drop of blood, mixing with it uniformly. This resulted in a sufficient dilution of red blood cells so that they appeared as separate, evenly distributed units, in a single layer. Also a sufficient degree of laking of the red cells occurred so that their outlines became faint, and the malaria parasites stood out in greater contrast. Best results were obtained when the combined drop of blood and water did not spread over the entire coverglass; when this occurred there often resulted a "floating" of the coverglass so that it moved with movements of the objective, making it difficult to focus upon a parasite. Good fixation of the coverglass was obtained when the diluted blood did not reach the periphery. Rimming with vaseline also provided additional

* Faucet water in other communities may contain constituents which render it unsuitable for use in the manner described here.

fixation of the coverglass and prevented evaporation; preparations made in this manner have been studied under the microscope for hours at a time.

Detection of malaria parasites in wet preparations and dry blood.

The two outstanding characteristics of *Plasmodium vivax* as seen under darkfield illumination are the brilliant refractiveness of the particles of pigment, and their active brownian movement. The index of refraction of these pigment particles is such that they manifest a sparkle or glitter which is not possessed by anything seen in a normal blood smear. Even when the source of light is reduced to the point where the outlines of the red blood cells are no longer visible, the glitter of the pigment is still visible. (See Figure 1.) When direct illumination is thrown upon the parasite it can be seen that the highly refractive particles are brownish-black in color, which is taken to indicate that they either represent or contain pigment. Other particles possessing less refractiveness are also present in the parasite, but the nature of these is uncertain. In the case of younger parasites, the pigment particles occupy only a small area of the red blood cell, and the parasite outline can readily be seen, often changing in outline as it undergoes ameboid movements. The particles of pigment are small in size and less refractile in young parasites, as compared to older parasites and gametocytes, (See Figure 3.)

The only other cells which might conceivably cause confusion are the leucocytes. These contain granules which are moderately refractive under darkfield illumination, and possess brownian motion as well. When a malaria parasite is seen in close proximity to a leucocyte, however, it is readily seen that there should be very little cause for error in identification. The leucocytes are larger in size, possess a single or lobulated clear area representing the nucleus, the granules are packed closely together without intervening clear areas, and the refractiveness of these granules is such that they are very pale in contrast to the bright pigment granules. In the malaria parasites, the pigment granules are separated by intervening clear areas, and are uniformly distributed throughout the cell, except in the case of *falciparum* gametocytes, which show characteristics which will be described later. The leucocytes have a "ground glass" appearance which is not possessed by malaria parasites. Almost immediately after blood is drawn, the leucocytes begin to demonstrate ameboid movements, with the extrusion of pseudopodia, and can frequently be observed engulfing malaria parasites. The latter can often be seen intact within a leucocyte, the pigment still undergoing

Figure 1

PLASMODIUM VIVAX. The darkfield illumination has been reduced to the point where the outlines of the erythrocytes are no longer visible, yet the highly refractile pigment of the parasite continues to glitter.

Figure 2

PLASMODIUM VIVAX, as observed under darkfield illumination. An unstained thin blood smear was used in this preparation.

Figure 3

PLASMODIUM VIVAX. The young ring stage shows considerably less pigment than the older parasite in the upper part of the field.

active brownian movement. This movement finally ceases and the pigment becomes dispersed throughout the cytoplasm of the leucocyte. The highly refractile nature of the pigment continues to preserve its identity within the leucocyte, distinct from the cytoplasmic granules of the latter. (See Figure 4.)

A dried thin blood smear, prepared in the usual fashion, may also be examined under darkfield illumination. The brownian movement will, of course, be absent, but the highly refractile pigment will still stand out in sharp contrast to anything else seen in the field. Figures 2 to 4 show the appearance of *vivax* parasites, in unfixed, unstained dry blood smears. Such a method may be employed for delayed examination, when a darkfield microscope is not available at the place where the blood is drawn, as, for example, in the patient's home.

From a diagnostic standpoint, the question arises as to the relative time relationships for obtaining positive results by the above methods as compared to the stained, thick-drop method. In a number of cases where such time relationships were compared, it was observed that the thick drop was usually reported as positive on the

Figure 4

The difference between the refractiveness of the pigment granules of the malaria parasite and the granules of the leucocyte in the lower right hand corner is readily evident. The latter has a "ground glass" appearance.

day prior to the detection of parasites by any of the darkfield illumination methods described above, in the case of primary attacks of *vivax* malaria. However, in the case of relapses of *vivax* malaria, where a high parasite count, and large parasite forms, are usually present at the onset of clinical manifestations, no difficulty was encountered in detecting parasites by darkfield methods with the first attempt. Since much of the malaria occurring in troops returning to this country after service in malarious areas will be relapsing *vivax* infections, this method may be useful in diagnosing them. On our malaria therapy service, this method has been utilized on a number of occasions for the rapid diagnosis of relapses in patients who had previously received sporozoite-induced infections, and requested readmission for a febrile condition believed to be a relapse. It was possible, within the few minutes needed to prepare a wet preparation and examine it, to determine in some instances that the etiology was not malaria, and thereby prevent an unnecessary admission to the service. It would appear that the simplicity of this procedure, as well as the rapidity with which results are available, should make it a useful laboratory technique, particularly where frequent cases of relapsing *vivax* malaria are encountered.

As would be expected, this method does not lend itself to the diagnosis of *falciparum* malaria, as the tiny rings found in the peripheral blood do not contain sufficient pigment to be readily visible under darkfield illumination. Gametocytes of this species are readily seen with darkfield illumination, but since they appear late in the disease, their presence is of little early diagnostic value.

Exflagellation Studies

The darkfield illumination method lends itself very well to observations of exflagellation. The commonly used fixed, stained smear method has often failed in our hands, probably due to the fact that fixation must be carried out near the precise moment that exflagellation occurs in order to demonstrate this process successfully. Since the process of exflagellation covers a short period of time, seconds rather than minutes in duration, fixation at the proper moment is more often an accidental, than a usual phenomenon. By means of the wet smear preparation and darkfield illumination, the course of events preceding and following exflagellation is readily seen, and the results are consistent enough so that the influence of various drugs upon exflagellation can be studied. Exflagellation has been regularly observed in both *vivax* and *falciparum* infections. When a drop of water is added to an equivalent-sized drop of *vivax* blood, as mentioned above, the exflagellation process occurs in a surprisingly short time, usually within five minutes. Before it was

realized that exflagellation in hypotonic environment occurred in such a short time, this event was frequently missed, due to delay in examining the preparation. When the preparation was placed under the microscope immediately after the blood was drawn, little difficulty was encountered in demonstrating exflagellation when the stained smear revealed the presence of male gametocytes.

The high-power dry objective was found to be more satisfactory than the oil-immersion objective in searching for exflagellating gametocytes, since a larger field containing many parasites could be observed. Usually the first evidence of exflagellation is a rippling movement of the red blood cells in the vicinity of the gametocyte, as its flagellae lash these cells. Flagellae can readily be seen by the experienced eye under the high-power dry objective, and following their separation from the parent cell can often be seen moving amongst the red blood cells, looking not unlike spirochaetes of the *borrelia* type.

If a change is made to oil-immersion at the first evidence of exflagellation, the process can be observed at higher magnification and in greater detail. The large granules of pigment in the gametocytes undergo rapid movement prior to exflagellation. This movement is so vigorous that frequently protusions of the parasite wall are seen under the impact of these masses. The activity of the flagellae

Figure 5
Gametocyte of PLASMODIUM FALCIPARUM.

prior to piercing the parasite wall may contribute to this movement of pigment. As exflagellation occurs, there is a tendency for the pigment to arrange itself in concentric form in the center of the parasite mass. Subsequent to exflagellation, the brownian movement of pigment becomes slower, and eventually ceases, as if the viscosity of the intracellular medium had been altered.

The exflagellation process observed in *falciparum* malaria follows a course similar to that seen in *vivax* malaria. In the crescent-shaped early stages the pigment granules of the gametocyte are at first congregated toward the center of the parasite, and are immobile (Figure 5). Within a short time, a spherical form is assumed by the gametocyte and the pigment becomes active in brownian movement, as mentioned above for *vivax* gametocytes. The subsequent course of events is as described above.

Observations of the direct action of antimalarial drugs upon parasites.

These observations are of a preliminary nature, and further investigations are now in progress. There is substantial evidence that a direct effect is produced by drugs on *P. lophurae* (Hewitt and Richardson, 1943). Observations made of the influence of various antimalarial drugs *in vitro* upon the appearance of parasites and upon the brownian movement of pigment particles, as seen under darkfield illumination, will be mentioned here. In parasitized blood which is maintained at room temperature, brownian movement of pigment particles in *vivax* parasites can be observed for as long as 72 hours after the blood is drawn and citrated. However, after a period of approximately 24 hours, parasites begin to develop a crinkled, granular appearance, and the pigment particles become motionless. These changes are observed in increasing numbers of parasites beyond this period. For this reason, observations were limited to a 24-hour period after the blood was drawn, to avoid confusion with normal degenerative changes.

Atabrine, plasmochin, and quinine respectively were added in various concentrations to parasitized, citrated blood containing *vivax* parasites and observed under darkfield illumination for a period of 24 hours. It was noted that concentrations of these drugs of the order of those obtained in plasma following oral administration at therapeutic levels produced no demonstrable effect upon the appearance of the parasite or the brownian movement of pigment, in comparison with an untreated control sample. For this reason, high concentrations of these drugs were subsequently used, as, for example, 50 mg. per cent quinine, 1 mg. per cent atabrine, and 10 mg. per cent plasmochin. While these concentrations are from 10 to 1000 times greater than those observed *in vivo*, they did not appear

to devitalize leucocytes, which continued to manifest brownian movement of their granules and active progression through the field. No appreciable difference was noted in the appearance of malaria parasites and the brownian movement of pigment particles under the influence of these high drug concentrations over a period of 24 hours, when compared to an untreated control sample. Toward the end of the 24 hour period, degenerative changes began to appear in all samples, but the ratio of parasites with active pigment to those containing immobile pigment remained approximately the same for all specimens, regardless of the drug employed. Under the conditions of these experiments, it was therefore not possible to demonstrate a direct effect of high concentrations of antimalarial drugs upon malaria parasites, when the appearance of parasites under darkfield illumination was used as a basis for study. This does not imply, of course, tht such a direct effect does not take place.

Observations are in progress with regard to changes observed in *vivax* parasites under darkfield illumination following the oral administration of antimalarial drugs. Following the administration of atebrine in such infections, clear spaces free of pigment, presumably vacuoles, appear in the larger forms and there is a tendency for pigment granules to aggregate in clumps and lose their brownian movement. Further study is in progress relative to changes occurring under the influence of various drugs and their relationship to changes observed *in vitro*.

Summary

1. The apparatus and technic employed for studying both wet and dry preparations of blood containing malaria parasites under darkfield illumination is described.

2. The distinctive features of *vivax* parasites as observed under darkfield illumination are the refractiveness of the pigment, and its active brownian movement.

3. This technique would appear to be useful as a diagnostic instrument, in cases of relapsing *vivax* malaria. It is not as sensitive as the stained thick-drop method during the early days of the primary attack, nor is it of value in the diagnosis of *falciparum* malaria prior to the appearance of the gametocytes.

4. The exflagellation of *vivax* and *falciparum* gametocytes can be observed readily under darkfield illumination, and with the technique employed occurs within five minutes after the blood is drawn.

5. Preliminary observations of the effects of high concentrations of antimalarial drugs *in vitro* upon the appearance of *vivax* parasites under darkfield illumination failed to reveal significant changes, when comparison was made with untreated blood.

6. The simplicity of this technique, together with the rapidity with which *vivax* parasites can be detected when present in sufficient numbers, may justify its routine diagnostic use.

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DESCRIPTION OF *CHAGASIA ROZEBOOMI*, AN ANOPHELINE FROM CEARÁ, BRAZIL*

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Three of the four recorded species of *Chagasia* have been collected by the authors in Brazil. There are *Chagasia fajardoi* Lutz, 1904, occurring in the central, eastern and southern sections of the country, *Chagasia bonneae* Root, 1927, encountered in the Amazon region, and *Chagasia rozeboomi* Causey, Dean and Dean, 1944, collected in a mountainous area in the northeast. *Chagasia bathanus* Dyar, 1928, has not yet been reported from Brazil but is known to occur in Central and South America as far south as Venezuela.

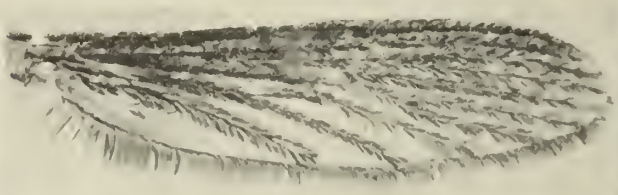
Chagasia rozeboomi was found in high wooded areas at Londa, near Crato, state of Ceará, Brazil, approximately 500 meters above sea level. It has not yet been encountered outside the type locality. A total of 44 larvae, 14 pupae and 140 adult females was collected between July and September, 1941 and in January, 1944. From the larvae four males and several females were bred out and 25 ovipositions were obtained from the wild females.

The larvae were found in shaded forest springs or in the streams arising from them, in clear, cool, moving water with some marginal vegetation and thick deposits of fallen leaves. Larvae and pupae were found in small numbers and usually unaccompanied by other anophelines. On one occasion larvae of *Chagasia rozeboomi* and of *Anopheles argyritarsis* were found in the same shaded spring.

Adults rarely if ever enter houses. They were captured outdoors, usually at sunset, although one specimen was taken while feeding at 4 p. m. During a crepuscular capture made simultaneously on man and horse, seven females were collected on man and none on the horse, but in other captures, when only a horse was used as bait, many specimens were obtained .

Chagasia rozeboomi is readily separated from *Chagasia bonneae* and *Chagasia bathanus* in the larval and adult stages. In adult coloration and genitalic characteristics it is closely related to *Chagasia fajardoi* but can be easily distinguished by striking larval and egg differences.

* The studies herewith reported were begun as a part of the program of the *Servica de Malaria do Nordeste* maintained jointly by the Ministry of Education and Health of Brazil and the International Health Division of The Rockefeller Foundation, and completed as a part of the program of the *Serviço Especial de Saude Publica* maintained jointly by the Ministry of Health and Education of Brazil and the Office of the Coordinator of Inter-American Affairs.



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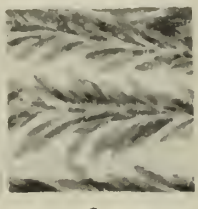

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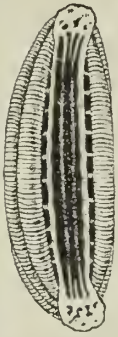

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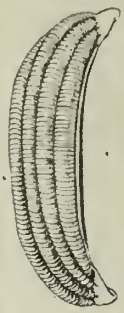

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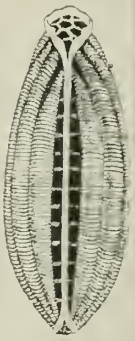

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Plate I

Fig.	1	—*C. rozeboomi.*	Wing.
Fig.	2	—*C. rozeboomi.*	Detail of wing showing shape of scales.
Fig.	3	—*C. rozeboomi.*	Last hind tarsal segments.
Fig.	4	—*C. rozeboomi.*	Eggs.
Figs.	5 and 6	—*C. rozeboomi.*	Eggs. Camera lucida drawings.
Fig.	7	—*C. rozeboomi.*	Male terminali.
Fig.	8.	—*C. fajardoi.*	Egg. Camera lucida drawing.

Description of *Chagasia rozeboomi*

Adult female. Mosquito of medium size. Antennae pilose, with patches of dark scales at tips of segments except last 2 or 3. Palpi covered with black erect scales; few pale scales on apex of segments III, IV and V, scattered or grouped, but not forming definite rings.

Mesonotum with brown integumen and with pale, truncate scales, more numerous, whiter and larger on anterior half, chiefly arranged around two dark, bare, small, submedian spots; scales on posterior half of mesonotum smaller, yellowish; many long, black or dark brown, erect scales with truncate tips on lateral margins. Scales and bristles present on pro-epimeron. Scutellem slightly trilobed.

Wings unspotted; scales elliptical, all of about the same type and same brown color (Plate I, figs. 1 and 2). Femora and tibiae predominantly dark brown, speckled with pale, irregular spots of yellowish scales. Front tarsi: segment I dark brown with small pale spots or rings; segments II and III dark brown with basal and median pale ring; segment IV pale at basal half, remainder dark brown; segment V dark brown with inconspicious pale scales at base. Mid tarsi: segment I dark, with many pale spots or rings, apex dark; other segment as on front tarsi. Hind tarsi: Segment I with 6 or 7 white rings, closer on basal half, separated by broader black bands on distal half; apex black. Segments II, III and IV white, with apical 1/4 to 1/3 black; segment V nearly all white, with black apical ring usually measuring 1/5 or less of segment (Plate I, fig. 3).

Abdomen covered with brown hairs, scales absent.

Egg. The egg has been described and figured in a previous paper by the authors (1944). Strongly convex ventrally and concave dorsally. About six or eight parallel floats tightly approximated over surface of egg except for broad longitudinal dark band with faint reticulated pattern enclosed by frill at dorsal surface, narrow longitudinal bands of distinct reticulated exochorion at sides of frill and broader at ventral surface. Each extremity of egg surmounted dorsally by thick, whitish, spongy cap-like structure (Plate I, figs. 4, 5 and 6). In some specimens one or more floats interrupted instead of extending from tip to tip of egg.

Larva. Preclypeal hairs small, widely separated. Inner anterior clypeal hairs widely separated, long with strong stem and many long conspicious, simple lateral branches arising from apical three quarters. Outer anterior clypeal hair similar and situated posterior to inner hairs. Posterior clypeal hairs similar and inserted much behind (Plate II, fig. 11). Frontal head hairs small, with about 5 to

Table 1.—Differential characters of known species of *Chagasia*

Characters	*C. fajardoi*	*C. bonneae*	*C. bathanus*	*C. rozeboomi*
Adult				
Wing scales	All brown, long, lanceolate	Intermixed dark and light; dark are shorter, broad, with rounded or blunt tip; light are longer, lanceolate	As in *bonneae*	As in *fajardoi*
2nd, 3rd and 4th hind tarsal segments	White, each with one apical black band	As in *fajardoi*	With additional, narrow black, sub-basal ring on segments 2 and 3	As in *fajardoi*
5th hind trasal segment	Basally white, with apical 1/4 to 1/3 black	Nearly all black with white basal ring when seen from one side; nearly all white with black apical ring when seen from other side.	As in *fajardoi*	Nearly all white with black apical ring usually measuring 1/5 or less of segment
Black on 3rd and 4th hind tarsal segments	On 3rd wider than on 4th	On 3rd narrower than on 4th	———	As in *fajardoi*
Male Terminalia				
Internal lobe of side piece	With 2 stout spines	Spines many, stout	As in *bonneae*	As in *fajardoi*
Egg	One extremity with large, dorso-apical collar surrounding reticulated area; other extremity without differentiated frill or with very small collar apically placed. Frills closely approximated at median line.	Unknown	Unknown	Both extremities surmounted dorsally by white, spongy, cap-like structures of about same size and shape. Frills widely separated at median line

Table 1.—Continued

LARVA Frontal head hairs	large; inner pair inserted much beyond median paid with many (about 12-14) branches, surpassing anterior margin of clypeus	Large; inner pair inserted slightly anterior or at same level as median pair, with many branches, almost reaching or surpassing anterior margin of clypeus	As in *fajardoi*	Very small; inner pair inserted much beyond median pair, with about 5 to 8 branches, not reaching base of posterior clypeal hairs
Inner hair of submedian prothoracic group	Leaflets long, with moderately notched (or smooth) margins after middle, and long tapering, almost filamentous tips	Leaflets long, with long tapering, almost filamentous tips, most with smooth margins, some with few serrations	As in *bonneae*	Leaflets short, tips notched or frayed, not filamentous
Median hair of submedian prothoracic group	Long, one and one half to twice as long as inner hair; bifid, the two long bifurcations provided each with many hair-like branches	As in *fajardoi* but usually longer, more slender	As in *bonneae*	Short, slightly longer than inner hair; bifid, the two short, stout branches developing into leaflets notched or frayed at tip.
Palmate hairs of abdominal segments I and II	Leaflets broad, most with notched margins	Leaflets narrow, all or almost all with smooth margins	As in *bonneae*	As in *fajardoi*
Palmate hairs of abdominal segments III to V.	Apical filament short, usually shorter than expanded portion	Apical filament very long, much longer than expanded portion	As in *bonnea*	As in *fajardoi*

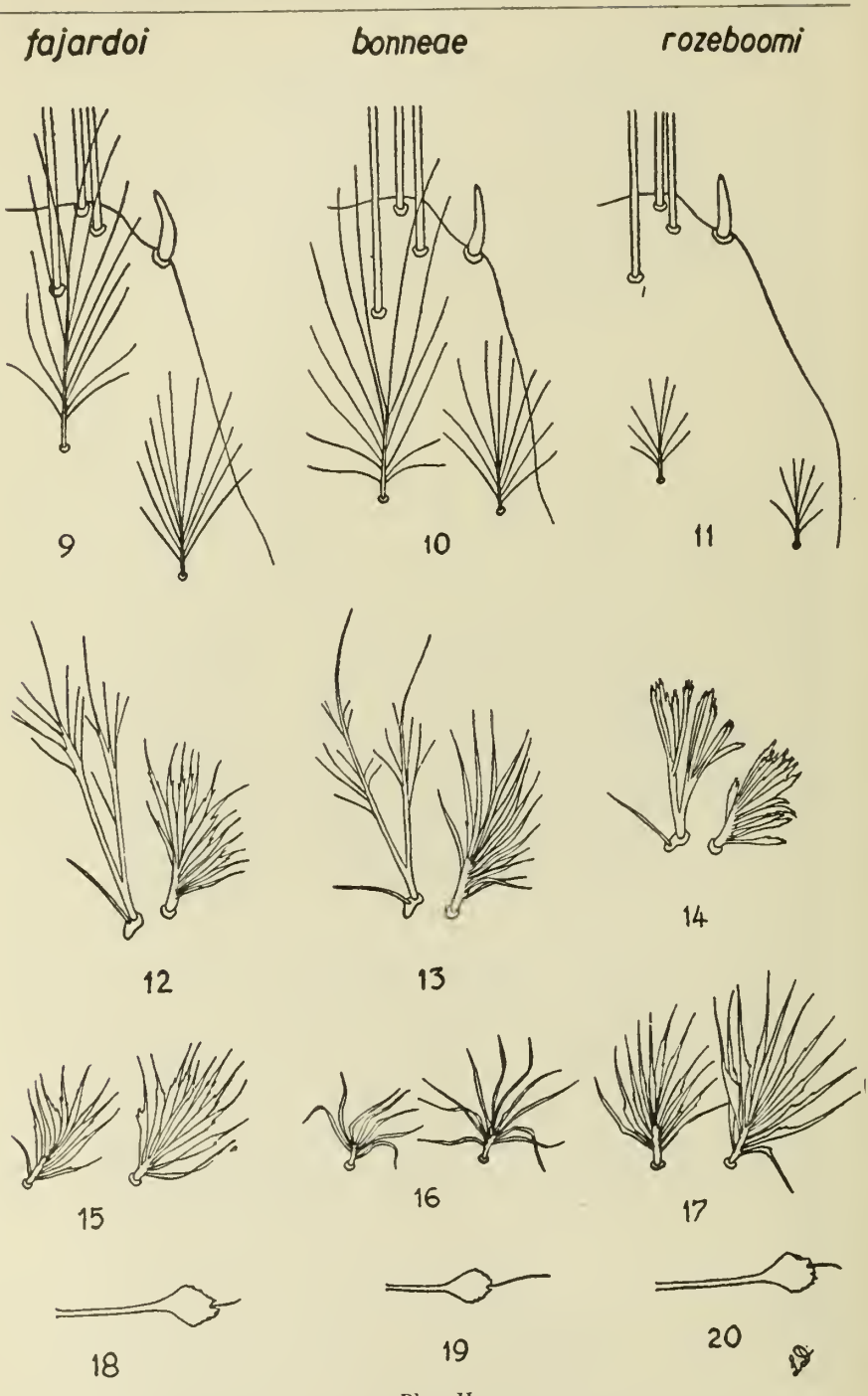

Plate II

Camera lucida drawings of fourth stage larvae, all to same scale.

Fig. 9 — *C fajardoi.* Detail of head showing inner and median frontal hairs.
Fig. 10 — *C. bonneae.* Detail of head showing inner and median frontal hairs.
Fig. 11 — *C. rozeboomi.* Detail of head showing inner and median frontal hairs.

8 branches; two pairs inserted on clypeus, the inner pair much anterior to median pairs; tips not reaching base of posterior clypeal hairs (Plate II, fig. 11); outer pair inserted close to subantennal hair.

Outer pair of submedian prothoracic group unbranched, inserted on base of median hair. Median hair of unusual type; stem bifid near base, the two short stout branches developed into leaflets with notched or frayed tips. Inner hair of group with thick stem and notched leaflets arising mostly from one side of stem and usually with short serrated tips (Plate II, fig. 14). Ventral and lateral surface of throax densely clothed with small hairs.

Lateral hairs of abdomen long and feathered on segments I and II; segments III to VI with small branched hairs. Palmate hairs on two first abdominal segments composed of flattened leaflets with irregular margins and narrow tips (Plate II, fig 17); on segments III to V racket-shaped with expanded portions much shorter than stem, irregular serrations on apical margins and short filament, usually shorter than expanded portion (Plate II, fig. 20). Anterior tergal plates well chitinized, small, more or less rounded or polygonal, with longitudinal axis almost same length as transverse axis. Tergal plate of segment VIII broader and shorter than preceding one. Each abdominal segment with dorso-lateral areas covered with small hairs; lateral and ventral surface of abdomen also covered with small hairs. Spiracular apparatus with peculiar appearance of genus. Anterior flap prolonged into whip-like process, stem of whip represented by long appendage about 1/3 of total length, at tip of which is articulated long hair representing lash of whip. Posterior flap when viewed by high power lens shows lateral margins fringed with flattened hairs. Pecten with about ten teeth, two terminal long and eight median, short.

Pupa. Breathing trumpets with wide opening, margin with depression at about middle of posterior portion; two posterior angles almost similar (Plate III, figs. 22 and 23.

Hair A and C of abdominal segments V to VII represented by strong conspicuous spines; hair A on segments II to IV represented by thin, small spines in contrast to much longer and stronger hair C. On segment VIII hair C absent. In all segments except V and VI hair C much longer than hair A; on segment V hair A decidedly longer and on segment VI both of about same size, but hair A frequently slightly longer (Plate III, fig. 21). Posterior margin of abdominal segments dentate. Paddle ovate, with small but strong teeth on outer margin, smaller close to apex and on apical third of inner margin; basal two thirds of inner margin smooth; terminal paddle hair represented by short, strong and straight spine; accessory paddle hair very small and inconspicious, single or bifid

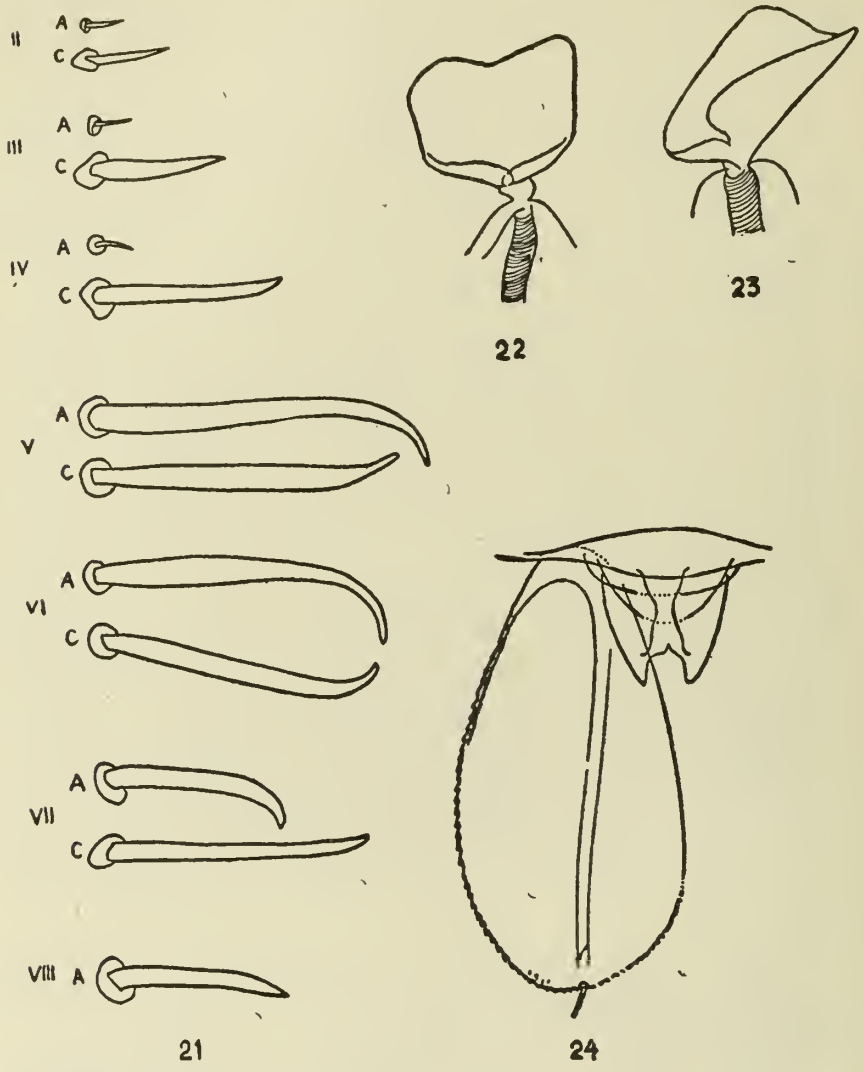

Plate III.—Camera lucida drawings.

Fig. 12 — *C. fajardoi.* Hairs of submedian prothoracic group.
Fig. 13 — *C. bonneae.* Hairs of submedian prothoracic group.
Fig. 14 — *C. rozeboomi.* Hairs of submedian prothoracic group.
Fig. 15 —*C. fajardoi.* Palmate hairs of abdominal segments I and II.
Fig. 16 — *C. bonneae.* Palmate hairs of abdominal segments I and II.
Fig. 17 — *C. rozeboomi.* Palmate hairs of abdominal segments I and II.
Fig. 18 —*C. fajardoi.* Leaflet of palmate hair of abdominal segment IV.
Fig. 19 — *C. bonneae.* Leaflet of palmate hair of abdominal segment IV.
Fig. 20 — *C. rozeboomi.* Leaflet of palmate hair of abdominal segment IV.
Fig. 21 — *C. rozeboomi.* Pupa. Spines A and C of abdominal segments.
Figs. 22 and 23 — *C. rozeboomi.* Pupa. Breathing trumpets.
Fig. 24 — *C. rozeboomi.* Pupa. Paddle.

(Plate III, fig. 24).

Male genetalia. Side piece short and broad; internal lobe small, bearing two distinct, stout spines and numerous long hairs. Claspette lobes rudimentary, uniformly clothed with numerous short, fine hairs, interspersed with few distinctly longer hairs. Mesosome slim, tube-like (Plate I, fig. 7).

Characteristics for the differentiation of the known species of *Chagasia* have been assembled in Table 1. The characters given for *Chagasia bathanus* are taken from the literature (Gabaldon, Herrera, Perez-Vivas and Rausseo, 1940; Komp, 1942). The other species are described from material collected by the authors. Some of the characters, however, were mentioned by previous authors (Gabaldon, Herrerea, Perez-Vivas and Rausseo, 1940; Peryassu, 1908; Root, 1927; Shannon and Del Ponte, 1927; Galvao and Barreto, 1938). The pupal characters are omitted from the table because they have not as yet been adequately studied in all species. The eggs of *Chagasia bonneae* and *Chagasia bathanus* are not included as they are unknown.

Chagasia rozeboomi is named in honor of Lloyd E. Rozeboom, medical entomologist at Johns Hopkins University School of Hygiene and Public Health, who kindly examined Doctor Root's slides of *C. fajardoi* to confirm differences between these specimens and the new species. Type specimens are deposited in the National Museum in Washington, D. C., U.S.A.

Summary

Chagasia rozeboomi, a new anopheline from Ceará, Brazil, was first recorded by the authors in a paper treating with anopheline eggs. The description and illustration of all stages of the life cycle of *Chagasia rozeboomi* and table of differential characters for the identification of the four known species of the genus *Chagasia* are presented.

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